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by

Morgane NENNIG
Born on 24 July 1993 in Thionville (France)

POPULATION STRUCTURE AND PHENOTYPICAL TRAITS OF *CAMPYLOBACTER JEJUNI* CIRCULATING IN LUXEMBOURG

Dissertation defence committee

Dr. Catherine Ragimbeau, dissertation supervisor
Dr. Sc., Laboratoire National de Santé, Dudelange, Luxembourg

Dr. Odile Tresse, dissertation co-supervisor
Dr.-HDR, UMR-INRAE 1280 PhAN, Nantes, France

Dr. Stephanie Kreis, Chair
Professor, Université du Luxembourg, Belvaux, Luxembourg

Dr. François Meurens, Vice Chair
Professor, UMR 1300 BIOEPAR, Oniris, Nantes, France

Dr. Philippe Lehours
Professor, Centre hospitalier universitaire, Bordeaux, France

Dr. Olivier Vandenberg
Professor, Ecole de Santé Publique, Brussels, Belgium

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Population structure and phenotypical traits of *Campylobacter jejuni* circulating in Luxembourg

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Composition du Jury :

Rapporteurs avant soutenance :

Olivier Vandenberg	Professor, Université libre de Bruxelles, Bruxelles, Belgique
Philippe Lehours	Professor, Centre hospitalier universitaire, Bordeaux, France

Examinateurs :

Stephanie Kreis	Professor, Université de Luxembourg, Belvaux, Luxembourg
François Meurens	Professor, Oniris, Nantes, France

Directrices de thèse :

Odile Tresse	Dr-HDR, UMR-INRAE 1280 PhAN, Nantes, France
Catherine Ragimbeau	Dr. Sc., Laboratoire National de Santé, Dudelange, Luxembourg

Invités

Ann-Katrin Llarena	Associate Professor, Norwegian University of Life Sciences, Aas, Norvège
Cédric Laczny	Research scientist, Université de Luxembourg, Esch-sur-Alzette, Luxembourg

Affidavit

I hereby confirm that the PhD thesis entitled “Population structure and phenotypical traits of *Campylobacter jejuni* circulating in Luxembourg” has been written independently and without any other sources than cited.

Luxembourg, 14/03/2022 _____

NENNIG Morgane

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AA	Amino acid
AAC	Acclimated to aerobic conditions
AC	Aerobic conditions
AD	Allele difference
AFLP	Amplified Fragment Length Polymorphism
AMR	Antimicrobial resistance
ANOVA	Analysis of variance
AS	Aerosensitive
AT	Aerotolerant
AWC	Adjusted Wallace coefficient
BAPS	Bayesian analysis of population structure
BFI	Biofilm formation index
BHI	Brain heart infusion
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CDT	Cytolethal-distending toxin
CFU	Colony forming unit
cg	Core genome
CIDT	Culture-independent diagnostic test
CJIE	*Campylobacter jejuni*-integrated elements
CRISPR	Clustered regularly interspaced short palindromic repeats
CT	Complex type
DALY	Disability-adjusted life year
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
EAA / EU	European economic area / European union
ECDC	European center for disease prevention and control
EFSA	European food safety authority
EPS	Extracellular polymeric substances
FNR	Fonds National de la Recherche
FORS	Food-borne outbreak reporting system
FQ	Fluoroquinolones
GBS	Guillain-Barré syndrome
GIT	Gastrointestinal tract
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
HAT	Hyper aerotolerant
HGT	Horizontal gene transfer
I_A	Index of association
IBD / IBS	Inflammatory bowel disease / Irritable bowel syndrome
ISO	International organization for standardization
LAMP	Loop-mediated isothermal amplification
LIH	Luxembourg Institute of Health
LOS	Lipooligosaccharides
MAC	Microaerobic conditions

MALDI-TOF MS	Matrix-assisted laser desorption – ionization-time of flight mass spectrometry
Mbp	Millions of base pairs / Megabases
MFS	Miller Fisher syndrome
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
MOMP	Major outer membrane protein
MS	Member state
MST	Minimum spanning tree
NAAC	Non-acclimated to aerobic conditions
NADH / NAD^+	Nicotinamide adenine dinucleotide
NGS	Next generation sequencing
OD	Optical density
ORF	Open reading frame
PCE	Predominant clonal evolution
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PQ	Paraquat
QC	Quality control
RM	Restriction-modification
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Sheep abortion
SAAC	Semi-acclimated to aerobic conditions
SG	Second-generation
SNP	Single-nucleotide polymorphism
SOD	Superoxide dismutase
ST	Sequence type
TA	Toxin-antitoxin
TCA	Tricarboxylic acid cycle
TG	Third-generation
T3SS	Type III secretion system
T6SS	Type VI secretion system
UC	Unique combination
UK	The United Kingdom
UPGMA	Unweighted pair group method with arithmetic mean
UPW	Ultra-pure water
USA	United States of America
VBNC	Viable but non-cultivable
VTEC	Verotoxin-producing *E. coli*
wg	Whole genome
WGS	Whole genome sequencing
WHO	World health organization

Abstract

Campylobacter is the leading cause of bacterial gastroenteritis worldwide. The most prevalent species, *C. jejuni*, is a strict microaerobic, capnophilic, and thermotolerant pathogen. Given its growth requirements, the ability of *C. jejuni* to persist in food environments and be transmitted throughout food processing has long puzzled scientists.

This study aimed to compare the different genetic profiles of *C. jejuni* strains, isolated in Luxembourg, at the core genome (cg) and whole genome (wg) levels to elucidate its genetic population structure. Using phenotypical assays in controlled conditions and functional genomics analyses from wgMLST data, the study also investigated the possible link between phenotypic traits and emergence or persistence of genotypes.

A high concordance in strain clustering was observed between genomic lineage classifications and the epidemic and endemic signals, regardless the three cgMLST typing schemes used. The higher genome stability within genomic lineages supports the hypothesis of a clonal expansion with monomorphic patterns over time and sources. A high correlation was observed between phenotypes and host-specific or generalist clonal complexes for oxidative stress, adhesion to abiotic surfaces, biofilm formation, and acclimation to aerobic conditions responses. These data allowed the establishment of metaphenotypes specific to the genomic lineages. Functional genomics analysis revealed factors that may contribute to the spatiotemporal survival of recurrent strains. These results also suggest the selection of better-adapted and persistent *C. jejuni* strains to environmental stresses throughout the transmission route to human.

Résumé

Campylobacter est la principale cause de gastro-entérite bactérienne mondiale. L'espèce la plus répandue, *C. jejuni*, est un pathogène microaérobie, capnophile et thermotolérant. De par ses exigences croissance, la capacité de *C. jejuni* à persister au sein des chaines de production alimentaire a longtemps intrigué les scientifiques.

Cette étude visait à comparer différents profils génétiques de *C. jejuni* isolés au Luxembourg, à partir du core genome (cg) et du génome entier (wg) afin de clarifier la structure génétique de sa population. En utilisant des tests phénotypiques en conditions contrôlées et des analyses de génomique fonctionnelle à partir de données wgMLST, le lien entre les traits phénotypiques et la persistance des génotypes a été exploré.

Le regroupement des souches en lignées génomiques est concordant avec des profils de type épidémique et endémique, indépendamment des schémas de typage cgMLST utilisés. La grande stabilité du génome au sein de ces lignées soutient l'hypothèse d'une expansion clonale de type monomorphique au fil du temps et de différentes sources. Les phénotypes sont corrélés aux complexes clonaux généralistes ou spécifiques à l'hôte pour les réponses au stress oxydatif, à l'adhésion aux surfaces abiotiques, à la formation de biofilms et à l'acclimatation aux conditions aérobies. Ces données ont permis d'établir des métaphénotypes spécifiques de ces lignées génomiques. L'analyse de génomique fonctionnelle a révélé des facteurs pouvant contribuer à la survie spatiotemporelle des souches récurrentes. Ces résultats suggèrent également une sélection de souches de *C. jejuni* mieux adaptées aux stress environnementaux le long des voies de transmission à l'Homme.

Chapter I: Introduction and objectives of the thesis

1. Discovery and taxonomy of *Campylobacter*

The first known observation of *Campylobacter* was performed by Theodor Escherich in 1886, who described spiral-shaped bacteria (Kist, 1986). Later in 1913, a microorganism causing abortions in sheep and cattle was identified and named *Vibrio fetus* (McFadyean and Stockman, 1913). However, the difference of phenotypic traits (e.g., G+C content of DNA or growth requirement) between *Campylobacter* and the type *Vibrio* species challenged the classification of this organism. Consequently, Sebald and Veron proposed a new genus and named it *Campylobacter* (Sebald and Veron, 1963). In 1938, in the United States of America (USA), a milk-borne outbreak of diarrhea was reported in prison, initially attributed to *V. jejuni*. In retrospect, with the name change, these were the first documented human cases of campylobacteriosis (Levy, 1946).

The *Campylobacter* genus belongs to the family *Campylobacteraceae*, of the order *Campylobacterales*, of the class *Epsilonproteobacteria*, and of the phylum *Proteobacteria* (Vandamme and De Ley, 1991). The development of new isolation techniques has led to a considerable increase in the number of *Campylobacter* species. Currently, 39 validly-published correctly named species of *Campylobacter* and 11 sub-species have been described (https://www.bacterio.net/genus/campylobacter, accessed 13.12.2021). *Campylobacter armoricus* and *Campylobacter massiliensis* are the last discovered and validated in 2019 and 2021 (Boukerb et al., 2019; Antezack et al., 2021). Among the 11 sub-species, two are related to *C. jejuni*: *C. jejuni* subsp. *jejuni* and *C. jejuni* subsp. *doylei* (Figure 1).

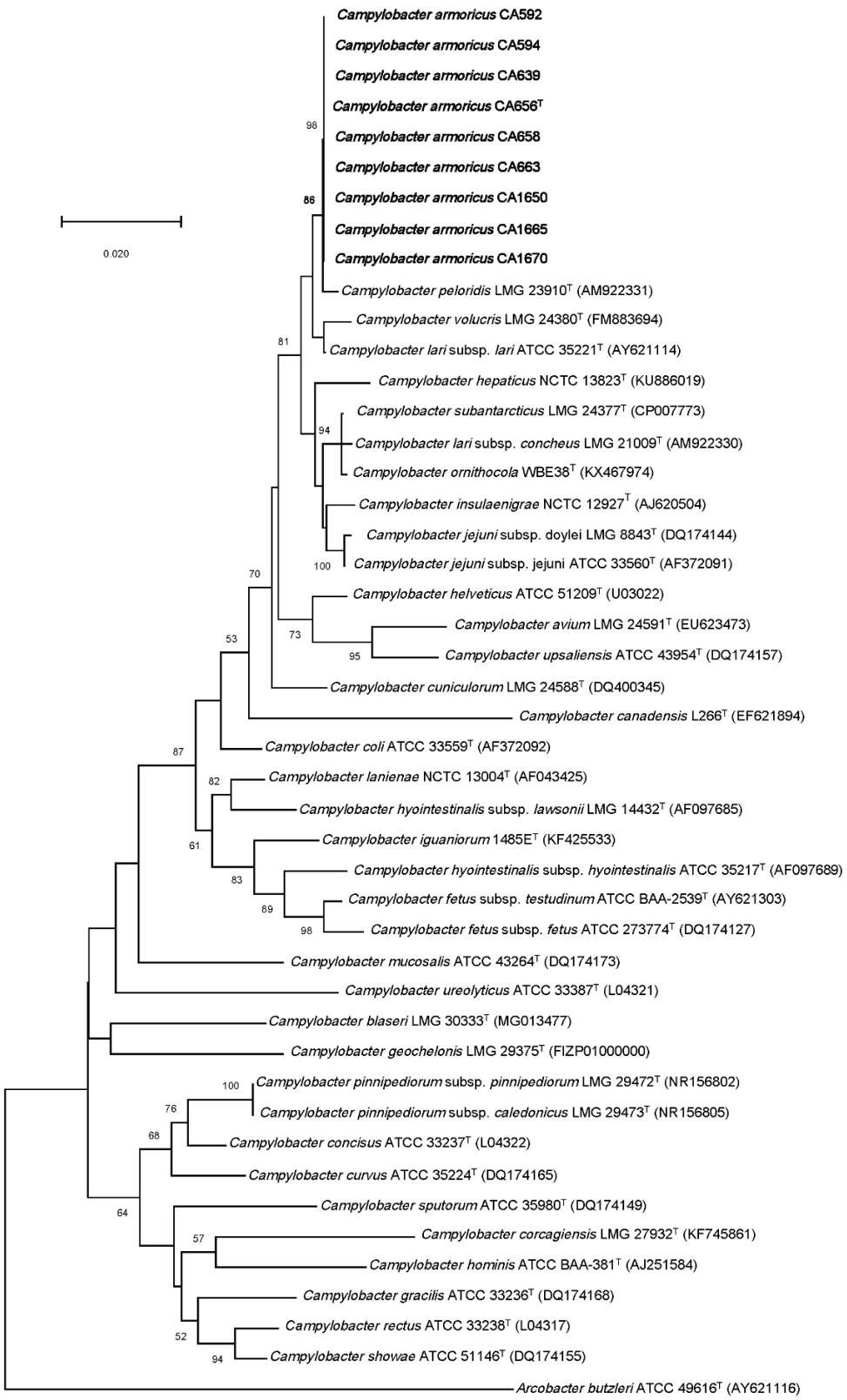

Figure 1: “Neighbor-joining phylogenetic dendrogram based on 16S rRNA gene sequences” (extracted from (Boukerb et al., 2019)).

2. Biology of *Campylobacter jejuni*

2.1. Physiological properties

2.1.1. Shape, motility and fitness

C. jejuni are curved or spirally gram-negative rods and have polar flagella, with a width of 0.2-0.8 µm and a length of 0.5-5 µm (Figure 2). They are motile with a characteristic rotating rapid corkscrew-like movement. *C. jejuni* is able to coordinate its flagella to swim in viscous environments, for instance (Vandamme, 2000; Cohen et al., 2020).

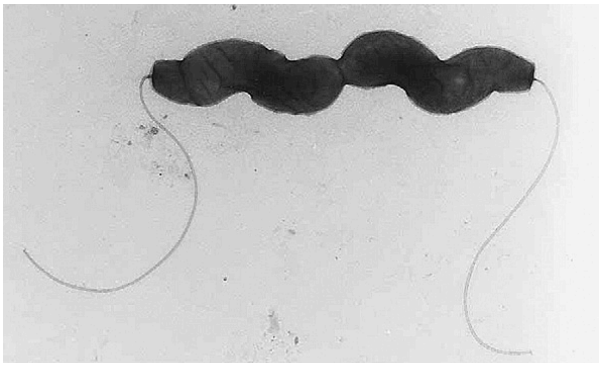

Figure 2: Photomicrography of *Campylobacter jejuni* in the process of dividing[1].

During continuous stress exposure, many bacteria can switch in viable but non-cultivable (VBNC) forms (Oliver, 2010; Pinto et al., 2015). *C. jejuni* possess this property and will change from a spiral motile form to a coccoid or filamentous form when subjected to unfavorable environmental conditions, such as heat or oxidative stresses (Rollins and Colwell, 1986; Tangwatcharin et al., 2006; Ikeda and Karlyshev, 2012; Ghaffar et al., 2015; Rodrigues et al., 2015). According to Griffiths, only the rod form allows an entry in the exponential growth phase, while filaments and coccoid forms are mostly involved in the survival strategy (Griffiths, 1993).

[1] Taken by Dr. Parker of Institute of Food Research, Norwich, UK.

2.1.2. Growth requirements

As a thermotolerant species, *C. jejuni* grows optimally at temperatures ranging from 37°C to 42°C (Table 1). Due to its microaerophilic and capnophilic nature, it requires an atmosphere impoverished in dioxygen (O_2; ~3-5%) and enriched in carbon dioxide (CO_2; ~10%) (Table 1). Moreover, its survival under atmospheric conditions (21% O_2, 78% N_2, and 1% other gas (CO_2, …)) is limited to a few hours (Garénaux et al., 2007; Hofreuter, 2014; Macé et al., 2015). *C. jejuni* is asaccharolytic, i.e., unable to metabolize carbohydrates, but it relies instead on amino acids and tricarboxylic acid cycle (TCA) intermediates for carbon source (Stahl et al., 2012; Hofreuter, 2014). Other optimum growth requirements, and their limits, are summarized in Table 1.

Table 1: Growth characteristics of *C. jejuni* species.

Parameters	Growth		
	Optimum	Growth limits	References
Temperature (°C)	42°C	31 – 45°C	(Hazeleger et al., 1998)
pH	6.5 – 7.5	5.5 – 8.0	(Doyle and Roman, 1981)
a_w	0.997	0.987	(Line, 2006)
NaCl (%)	0.5 %	2 %	(Doyle and Roman, 1982)
O_2	3 – 5 %	0 to 19 %	(Kaakoush et al., 2007)
CO_2	10 %	-	(Macé et al., 2015)

2.2. Survival and adaptation

2.2.1. General stress responses

These growth requirements limit the multiplication in food, unlike *Salmonella enterica* or *Listeria monocytogenes* (Park, 2002). Thus, the question arises about its ability to survive under stressful conditions *in situ* (i.e., on food and carcasses). Unlike other enteric pathogens, it is susceptible to different environmental stress conditions, lacking many adaptive responses. Its genome analysis highlighted the absence of the global regulator RpoS (Parkhill et al., 2000). This regulator governs the entrance in stationary phase an it is the basis for the survival of many Gram-negative bacteria during exposure to general stress (e.g., it controls nutrient scavenging abilities and stress response system) (Schellhorn, 2014). *C. jejuni* may develop survival mechanisms that could play a role in transmission through the environment, throughout the food chain, and host colonization to trigger human infection (Bronowski et

al., 2014; Yahara et al., 2017). These mechanisms mainly include protection against oxidative stresses in aerobic conditions, survival to heat shock, and adaptation to low pH in acidic conditions (Kim et al., 2021).

2.2.2. Oxidative stress response

Although considered highly susceptible to oxygen concentration, some strains of *C. jejuni* have been reported to develop different tolerance levels regarding survival in atmospheric air (~20% O_2 for 18 h) (Kaakoush et al., 2007; Rodrigues et al., 2015, 2016; Oh et al., 2017). It has been suggested that this ambient air exposure induces the formation of VBNC cells in *C. jejuni.* This essential survival strategy and physiological state is reversible as infectivity is still maintained in VBNC state and the infection process is activated after the resuscitation of VBNC cells (Oliver, 2010; Oh et al., 2015b).

According to Karki *et al.* (2018), one of the strategies explaining its persistence relies on its ability to cope with oxidative stresses (Karki et al., 2018). Oxidative stress results from the accumulation of highly reactive oxygen species (ROS) due to the incomplete reduction of oxygen. ROS include the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^\cdot$) (Imlay, 2008). Iron and oxidative stresses are intimately linked as shown in the Haber-Weiss reaction generating $HO^\cdot$ from H_2O_2 and O_2^- catalyzed by free iron ions (Figure 3) (Kehrer, 2000). The accumulation of hydroxyl radicals leads to the degradation and alteration of protein functions and causes irreversible damage to lipids and DNA (Fisher and Stadtman, 1992; Yamasaki et al., 2004). *C. jejuni* is exposed to highly variable dioxygen concentrations during its life cycle: throughout the food chain as well as in the host guts, as the immune system produces H_2O_2 to kill the microbes (Melo et al., 2019). Scavenging ROS during oxidative stress may be a key factor explaining its spread and survival in the environment (e.g., during the transmission of *C. jejuni* to food preparation surfaces) (Rodrigues et al., 2015).

2.2.3. The subsystem of ROS detoxication

The main systems defending cells against superoxides are mediated by superoxide dismutases (SODs), which catalyze the dismutation of superoxides into hydrogen peroxide and oxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) (Figure 3) (Touati, 2000; van Vliet et al., 2002). SODs are gathered in different classes according to their metal cofactors (e.g., copper-zinc, nickel, manganese, or iron) (Lynch and Kuramitsu, 2000). *Campylobacter* expresses a single iron-cofactored SOD: the SodB enzyme (Pesci et al., 1994).

Concerning the peroxides formed through the dismutation, they must be inactivated to prevent the formation of hydroxyl radicals. *C. jejuni* expresses several peroxidases, of which two are of high interest. First, *Campylobacter* expresses a single catalase enzyme, the heme-cofactored KatA enzyme, able to convert hydrogen peroxide to oxygen and water ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$) (Figure 3) (Grant and Park, 1995; Day et al., 2000). In *C. jejuni*, its expression is controlled by the PerR protein: repressed by iron, and induced by exposure to H_2O_2 (van Vliet et al., 1999; Palyada et al., 2009). The second *C. jejuni* peroxidase is the alkyl hydroperoxide reductase (AhpC), which converts alkyl hydroperoxides to the corresponding alcohols ($ROOH + NADH + H^+ \rightarrow ROH + NAD^+ + H_2O$) (Figure 3). According to Baillon *et al.* (1999), inactivation of the *ahpC* gene lead to an increased sensitivity to cumene hydroperoxide, but not to H_2O_2, suggesting that the two peroxidases have complementary functions, as in *Staphylococcus aureus* (Baillon et al., 1999; Cosgrove et al., 2007). Palyada and colleagues demonstrated that H_2O_2 reduction is primarily catalyzed by KatA and secondarily by AhpC (Palyada et al., 2009). Furthermore, inactivation of *ahpC* produced a mutant strain with a significantly reduced aerotolerance compared to the wild-type strain, suggesting that AhpC may contribute to the environmental survival of *C. jejuni* (Baillon et al., 1999). Finally, *C. jejuni* also encodes several other antioxidant enzymes, such as the thiolperoxidase Tpx and the bacterioferritin co-migratory protein Bcp. Bcp appears to be a peroxide reductase able to act on many compounds, whereas Tpx is a specific H_2O_2 detoxification enzyme; both play a role in protecting *C. jejuni* against oxidative stress but could also contribute to aerotolerance (Atack et al., 2008). Another bacterioferritin known as DNA protection during starvation protein (Dps), has been suggested to contribute to the oxidative stress response (Ishikawa et al., 2003). This protein can bind non-specifically to DNA in order to protect against stress caused by peroxides (Almirón et al., 1992). Furthermore, Dps is considered as an iron storage protein, which can minimize the effect of iron with oxidative stress to ensure bacterial survival (Ishikawa et al., 2003). According to Rodrigues *et al.* (2016), its higher abundance in aerobic conditions could be correlated with an adaptive response of *C. jejuni* to oxidative stress (Rodrigues et al., 2016).

C. jejuni lacks specific regulators well described in Gram-negative, such as OxyR or SoxRS, to control the regulation of detoxifying enzymes. However, it encodes other alternative response regulators such as CosR, Fur, and PerR. The *Campylobacter* oxidative stress regulator (CosR) belongs to the OmpR family of DNA-binding proteins, and it is a putative response regulator essential for the viability of *C. jejuni* (Hwang et al., 2011). However, it seems to be not exclusively dedicated to regulate enzymes of the sub-system of detoxification, as it could contribute to phase transition, biofilm maturation and macrolide efflux pump (Turonova et al., 2015, 2017; Guérin et al., 2020). The ferric uptake regulator (Fur) proteins controls iron

uptake in many Gram-negative bacteria, including *C. jejuni* (Wooldridge et al., 1994). In *C. jejuni*, it also regulates enzymes involved ROS scavenging (Atack et al., 2008). Initial characterization of the *C. jejuni fur* mutant highlighted also proteins with iron-responsive homeostasis regulation independent of Fur. Analysis of the *C. jejuni* genome allowed the identification of a Fur homolog, named PerR (van Vliet et al., 1998; Parkhill et al., 2000). The peroxide response regulator (PerR) is a repressor of genes encoding peroxide resistance enzymes (e.g., AhpC and KatA) by directly binding to their promoters. *C. jejuni* mutants are hyper-resistant to H_2O_2 by derepressing the transcription of *ahpC* and *katA* (van Vliet et al., 1999; Handley et al., 2015).

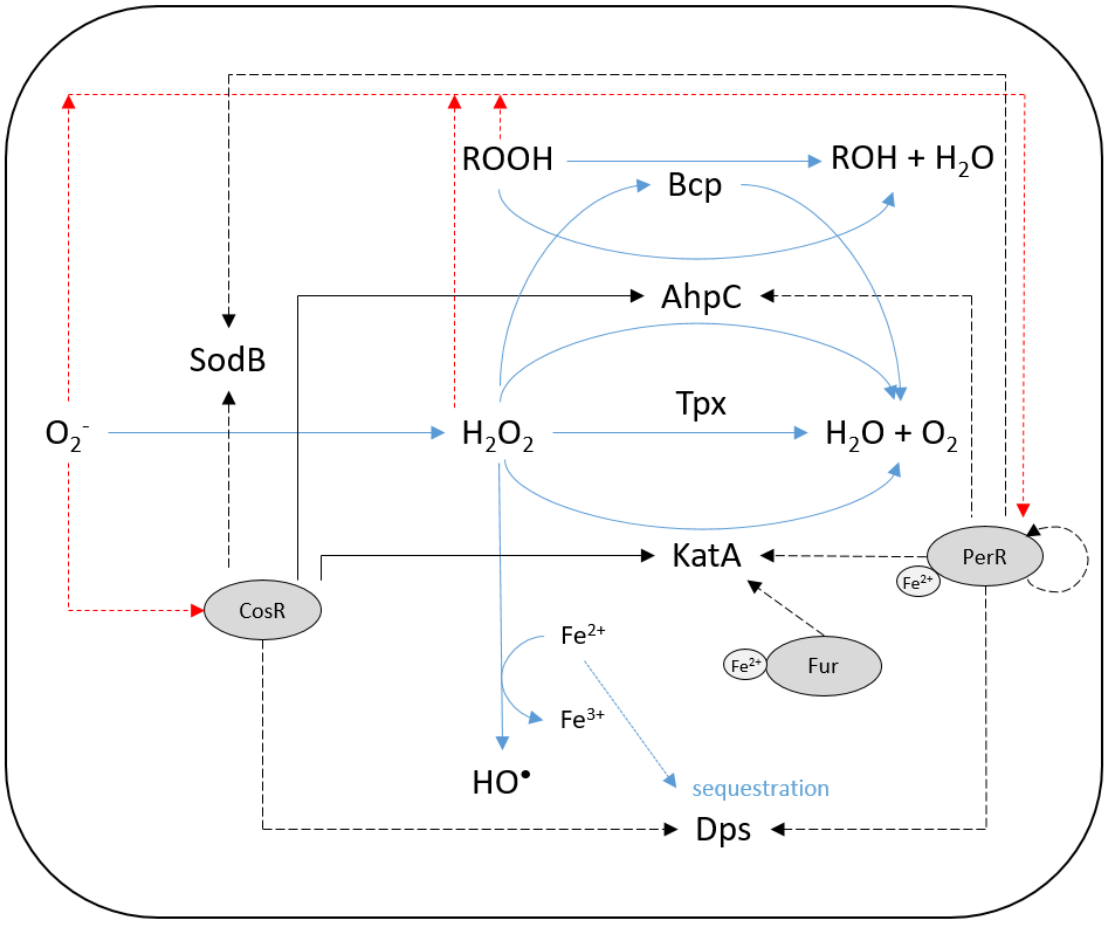

Figure 3: Schematic summary for the positive and negative regulations of enzymes and regulators implicated in oxidative stresses resistance in *C. jejuni* (adapted from (Kim et al., 2015)).

"Positive and negative regulations are indicated by black solid and dotted lines, respectively. Red dotted lines show transcriptional or translational down regulation by reactive oxygen species (ROS)" (extracted from (Kim et al., 2015)).

2.2.4. Acclimation to aerobic conditions

Aerotolerance contributes to the defense against oxidative stress, as exposure to oxygen results in ROS accumulation (Oh et al., 2015b). Different tolerance levels to atmospheric air have been shown to be strain-dependent (Kaakoush et al., 2007; Rodrigues et al., 2015, 2016; Oh et al., 2017). These aerotolerant profiles are frequently prevalent in chicken, and most of them belong to genotypes often implicated in human infections or even outbreaks (Oh et al., 2015a; Lee et al., 2019). The prevalence of this phenotypic trait among human clinical strains may be linked to an acclimation mechanism. While aerotolerance assays determine the survival capacity of strains after different exposure times to ambient air, acclimation to aerobic conditions determines the adaptive capacity of *C. jejuni* to multiply in atmospheric air. This latest characteristic involves physiological or morphological adjustments within the organism that improves its performance to cope with environmental alterations (Demmig-Adams et al., 2008).

Many studies are focused on the adaptation of microbial populations to degrade diverse compounds, such as organic waste (e.g., pyrene or different estrogens) or hydrocarbon contaminants (Mishamandani et al., 2016; Wang et al., 2018). They defined it as the process by which a microbial population adapts to break down a compound to which it is repeatedly exposed. It may involve three different mechanisms, including (i) the induction of enzymes or transports systems, (ii) the growth of a population of suitable microbes, or (iii) the evolution by the acquisition of mutations required for the adaptation (Knapp and Bromley-Challoner, 2003). More generally, adaptation is defined as the ability of an organism to adjust its phenotype to new environmental conditions throughout its lifetime, given its genotype. Adaptation is distinguishable from acclimation, which is defined as temporary beneficial modifications to an organism's phenotype caused by a change in its natural habitat without genome sequence alterations (Pinsky et al., 2013). Very few studies have investigated the ability of *Campylobacter* to acclimate to aerobic conditions. To the best of our knowledge, only one *C. jejuni* strain (out of ten tested) and one *C. coli* have been described in the literature as acclimated after several subcultures and multiplying under aerobic conditions (Rodrigues et al., 2015; O'Kane and Connerton, 2017).

2.3. Persistence in the environment

Bacteria developed several ways to persist in the environment as either intracellular or extracellular lives. Adhesion to inert surfaces and formation of biofilms are mechanisms described to date for *C. jejuni* extracellular survival in the environment. This mode of survival is widespread along all transmission

routes. As the intracellular survival is niche-specific to wastewaters and involves specific organisms (amoebas) (Snelling et al., 2008; Axelsson-Olsson et al., 2010; Thomas et al., 2010; Bui et al., 2012), this will not be treated here.

2.3.1. Adhesion to inert surfaces

Biofilm formation is a multistage process, starting with microbial adhesion, production and accumulation of an extracellular matrix (Flemming and Wingender, 2010). This initiation stage gathers the first and the second steps: adherence and adhesion. They are crucial and end with an irreversible attachment through covalent bonds (i.e., the adhesion) of cells to the substratum, usually taking place within the first hours of biofilm formation (Bryers, 2000). Several studies have highlighted the different potential adhesion of *C. jejuni*, which can adhere to various inert surfaces (e.g., stainless steel, fiberglass, coverslips, nitrocellulose membrane, and various plastics) and biotic surfaces (animal and human intestinal cells) (Kalmokoff et al., 2006; Asakura et al., 2007; Sanders et al., 2007; Pogačar et al., 2009, 2015). Many factors are involved in the adhesion process, such as the flagellum structure, which seems to be required to mediate adherence of *C. jejuni* strains (Svensson et al., 2014). Furthermore, it has been demonstrated that the over-expression of membrane proteins in oxygen-enriched conditions could enhance their adhesion to an inert surface (Sulaeman et al., 2012).

The 96-well microtiter plate technique is widely used to assess bacterial adhesion and biofilm formation (Azevedo et al., 2009). Based on this material, Chavant *et al.* (2007) have developed a more resolutive technique using magnetic beads, the BioFilm Ring test®, to assess biofilm formation of *L. monocytogenes, E. coli*, *S. carnosus*, and *S. xylosus* (Chavant et al., 2007). Sulaeman and colleagues performed comparative study with the biofilm initiation of *C. jejuni* and *C. coli* strains to an inert surface using this technique. They pointed out the higher adhesion capacity of *C. jejuni* strains isolated from food-processing environment or human clinical isolates from those originating from live animals (e.g., poultry or pork). These results suggested that food environment and human body could have applied a selective pressure to favor the strains with a greater adhesion (Sulaeman et al., 2010). As there was little information concerning the adhesion capacities of environmental isolates, Shagieva *et al.* (2020) compared the adhesion and biofilm formation abilities of isolates from various sources (surface and wastewater, food, and clinical isolates). Their work showed that adhesion capacity was comparable whatever the origin of the tested strains. Furthermore, according to their results based on a collection of 15 *C. jejuni*, it seems that the adhesion capacity is not correlated to the level of the biofilm formation ability (Shagieva et al., 2020).

2.3.2. Biofilm formation

According to Pascoe *et al*. (2015), the primary strategy used to persist in the environment by *Campylobacter* is the formation of biofilms (Pascoe et al., 2015). In broad terms, biofilms are defined as microcolonies of bacteria, enshrouded in a matrix of extracellular polymeric substances (EPS) and more frequently attached to surfaces (Donlan and Costerton, 2002). EPS are composed of various elements, including enzymes, nucleic acids, polysaccharides, and water. Biofilm development requires five successive coordinated steps: (i) the biofilm initiation including the reversible attachment of bacterial cells to a surface (adherence) followed by the irreversible attachment (adhesion), (ii) the biofilm growth involving the formation of micro-colonies, (iii) the maturation of the biofilm and (iv) the detachment and dispersion of the cells (Annous et al., 2009). *C. jejuni* is able to attach to inert surfaces, to form self/mixed-aggregates floating within a liquid culture, or to create a pellicle layer at the liquid-gas interface (Joshua, 2006). *C. jejuni* can form monospecies biofilm or coexist with other species in pre-established multispecies biofilms as a second colonizer (Hanning et al., 2008; Ica et al., 2012; Teh et al., 2019).

According to Flemming *et al.* (2016), a biofilm has a protective function through the matrix helping to reduce the impact of harsh environmental conditions (Flemming et al., 2016). For example, bacteria enclosed within it are a thousand times more resistant to antimicrobial agents than planktonic counterparts (Fux et al., 2005). Interestingly, the development of *C. jejuni* biofilms is more enhanced under aerobiosis rather than under optimal microaerobic atmosphere (Reuter et al., 2010; Sulaeman et al., 2012; Turonova et al., 2015). Thus, a favorable ecological niche for *C. jejuni* may be generated when cells are organized in a symbiotic relationship where dissolved dioxygen is presumably consumed by biofilm partners through aerobic respiration (Ica et al., 2012; Turonova et al., 2015). In the same way, *Campylobacter* biofilms in food processing plants shield this pathogen from cleaning and sanitizing measures. This critical point results in further contamination of food products and its spread in production lines (Nguyen et al., 2012; Brown et al., 2014). Therefore, bacterial biofilm is recognized as a crucial mechanism in the survival of *C. jejuni* in various ecological niches (García-Sánchez et al., 2019; Shagieva et al., 2020).

3. Genomic features of *C. jejuni*

3.1. Complete genome overview

The first whole genome sequence of *C. jejuni* was published in 2000 from the strain NCTC 11168. It is a circular genome composed of 1.64 Mbp, predicted to encode 1,654 proteins, with low G+C content (30.6%) (Parkhill et al., 2000). Rapidly, other genome sequences were publicly available, such as those of 81-176 or NCTC 11828 strains, and augmented information was implemented with new annotations (Hofreuter et al., 2006; Gundogdu et al., 2007; Pearson et al., 2007). Based on the available sequences, the average genome size was calculated and reached 1.6 to 1.8 Mbp, with a G+C content of approximately 32%. Up to date (07/10/2021), at least 273 *C. jejuni* complete genomes have been sequenced and deposited in Genbank (https://www.ncbi.nlm.nih.gov/nuccore), and over 40,000 draft genomes are available from the PubMLST database (https://pubmlst.org/).

Interestingly, hypervariable regions were identified linked to variations in short homopolymeric tracts commonly found in genes encoding biosynthesis, flagella or modification of surface structures (Parkhill et al., 2000). The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) type II system is present in *C. jejuni* and associated with the endonuclease *cas9* (CRISPR associated protein 9). CRISPRs are short sequences of repeats found in different bacterial genomes (Price et al., 2007a). In *C. jejuni*, a role of CRISPR-cas9 in the virulence was recently discovered: it appeared that multiple pathways involved in virulence are regulated by the CRISPR-cas9 system in *C. jejuni* NCTC 11168 (e.g., enhanced biofilm formation, higher adhesion and invasion, higher cytotoxin production, better motility) (Shabbir et al., 2018a). In addition, it seems to enhance antimicrobial resistance (AMR) in *C. jejuni* (e.g., upregulation of several ribosomal proteins expression) (Shabbir et al., 2018b).

C. jejuni have a set of conserved genes encoding essential housekeeping functions, i.e., the core genome, shared by all isolates from the same species, and a variable set of accessory genes, i.e., the accessory genome which is composed of partially shared and strain-specific genes (Lefébure et al., 2010; Segerman, 2012; Sheppard et al., 2012). Extensive research efforts have been focusing on the accessory genes, as they may be the key feature of the organism's variability in virulence, pathogenicity, and host-specificity (Epping et al., 2021). The accessory genome includes, for example, plasmids, integrated elements, and hypervariable regions. For instance, the 81-176 and RM1221 strains carry one to three plasmids (*pVir*, *pTet*, and *pCC31*), respectively. Whereas five *Campylobacter jejuni*-integrated elements (CJIE) (CJIE1-CJIE5), of prophage or plasmid origin, have been identified in RM1221 (Batchelor et al., 2004; Hofreuter

et al., 2006; Parker et al., 2006; Skarp et al., 2015). Four of these integrated elements carry genes encoding DNAses, themselves inhibiting the natural competence of *C. jejuni* (Gaasbeek et al., 2009b, 2010; Skarp et al., 2015).

3.2. Genome plasticity

C. jejuni is naturally competent, meaning that it can take up DNA from the environment (e.g., naked DNA without requirements of any particular treatment), and maximal competence was found in the early log phase of growth (Wang and Taylor, 1990; Young et al., 2007). Furthermore, competence varies between strains and lineages, being governed by the presence of genomic elements, such as restriction-modification (RM) system and methyltransferases. Indeed, it is selective in the DNA used in the transformation, as DNA methylation, induced by RM system and methyltransferases, is critical for this process (Beauchamp et al., 2017). A high number of RM systems was identified in *Campylobacter* (i.e., an average of 4 RM), considering its genome size (Vasu and Nagaraja, 2013). Environmental conditions could also influence *Campylobacter* competence: it seemed to be developed under microaerobic conditions with a pH between 6.5 and 7.5 and was nearly insensitive towards growth temperatures between 32 and 42°C, and CO_2 concentrations ranging from 0 to 50%. These conditions are close to the ones of the intestinal environment (Golz and Stingl, 2021).

This capacity leads to horizontal gene transfer (HGT) between genomes of different species and even more genetic diversity. They may occur during host colonization or *in vitro* growth, with plasmids and chromosomal DNA (Young et al., 2007). The new sets of genes are acquired by transduction, transposition, or transformation. In regards to the latest process, the bacteria-phage coevolution is known as a driver of ecological and evolutionary processes in microbial communities (Koskella and Brockhurst, 2014). For instance, some bacteria (e.g., *V. cholera*) depend on virulence or fitness factors encoded by specific prophages to cause a specific disease (e.g., cholera), while others (e.g., *S. aureus*, *S. enterica* serovar Typhimurium…) have a multitude of prophages, and each phage-encoded virulence or fitness factor contributes to the fitness of the bacteria (Brüssow et al., 2004). Some *C. jejuni* isolates also harbored CJIE1 prophage, whose presence was strongly linked to specific phenotypic behaviors (e.g., enhanced adherence and invasion abilities of host cells) (Clark et al., 2016). The high level of acquisition of novel genetic elements by HGT achieved through homologous recombination is, therefore, the major factor in the generation of genetic and antigenic diversity in *Campylobacter* (Sheppard et al., 2012).

Furthermore, a mutation rate was estimated for *C. jejuni* at 3.4 x 10^{-6} substitutions per site per year (s/s/y), being similar to the estimated one of its close relative *Helicobacter pylori* (~ 4.5 x 10^{-6} s/s/y), higher to the *Staphylococcus aureus* one (2.43 x 10^{-6} s/s/y), and much higher than the one determined for *Klebsiella pneumoniae* (2.99 x 10^{-7} s/s/y) and *Bordetella pertussis* (1.74 x 10^{-7} s/s/y) (Morelli et al., 2010; Duchêne et al., 2016; Calland et al., 2021). The presence and functionality of DNA repair mechanisms in *C. jejuni* are mainly unknown. Findings suggest that *C. jejuni* expresses some proteins functioning as DNA repair enzymes (Nth and Ung proteins acting as base excision repair enzymes) (Gaasbeek et al., 2009a; Dai et al., 2019). However, the absence of a functional mismatch repair pathway may underpin the genetic diversity of *C. jejuni* population (Dai et al., 2019).

4. Epidemiological data of campylobacteriosis

4.1. Human campylobacteriosis

Campylobacter is the leading cause of bacterial gastroenteritis worldwide (WHO, 2013). The related disease, so-called campylobacteriosis, has been the most frequently reported zoonosis in the European Union (EU) since 2005 (EFSA and ECDC, 2021a). Its incidence has increased throughout Europe, with a stabilization phase during 2015-2019 (59.7 cases per 100,000 inhabitants in 2019). In Luxembourg, an upward trend was also observed from 42.6 cases per 100,000 population recorded in 2005 to a peak of 158.8 in 2014. The numbers have remained relatively high with an incidence of 103.8 per 100,000 inhabitants in 2017 and 2018 (EFSA and ECDC, 2006, 2015, 2019) (Figure 4).

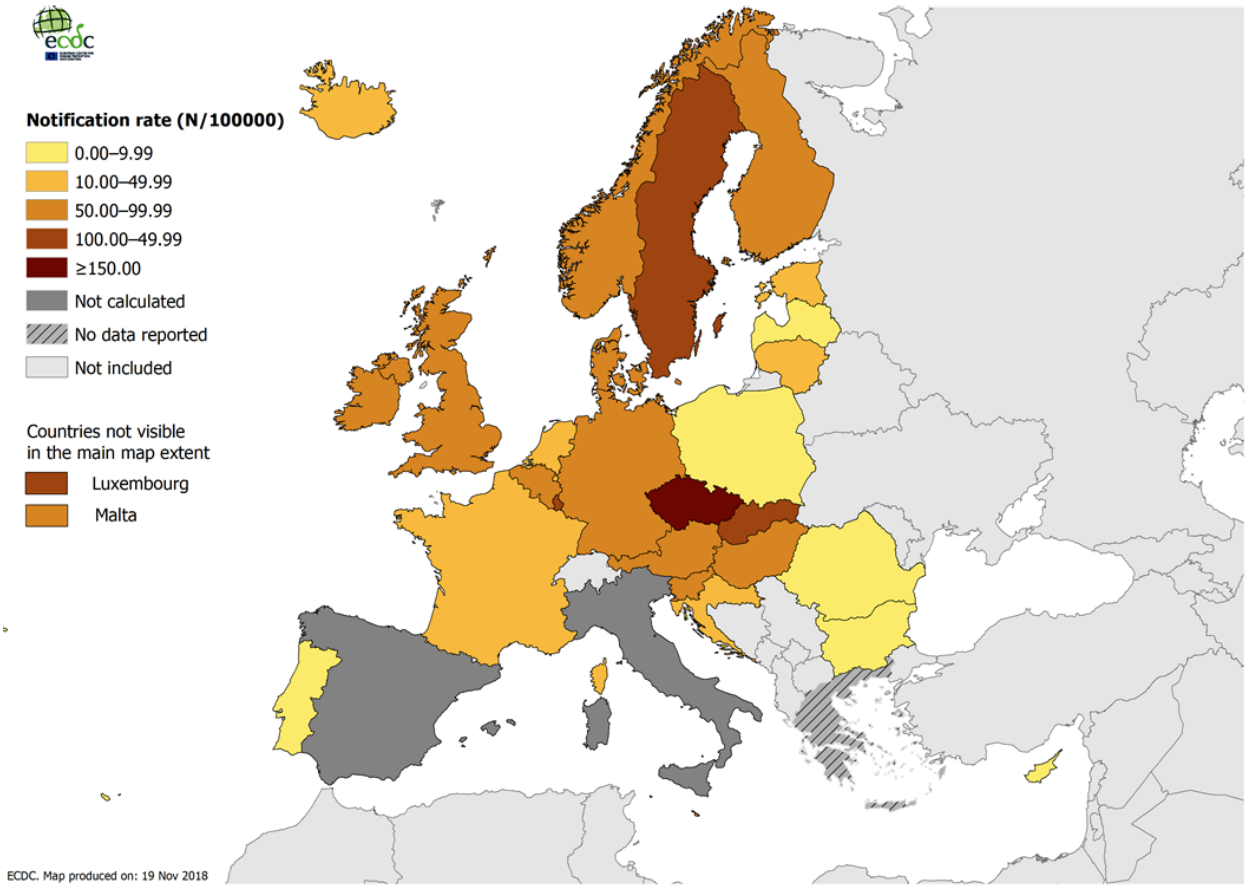

Figure 4: "Distribution of confirmed campylobacteriosis cases per 100,000 population by country, EU/EEA, 2017" (extracted from (ECDC, 2019a)).

A similar rising trend was observed for the incidence of campylobacteriosis cases in the USA since 2004 with an increase of 13% between the period 2016-2018 and 2019 (Geissler et al., 2017; Tack et al., 2020). This rise was also detected in Australia with a notification rate 4% higher (125 cases per 100,000 population in 2019) than the previous five-year average (Government of Western Australia, Department of Health, 2021). Furthermore, *Campylobacter* infections show a clear seasonality throughout the year, with a peak of cases in summer and minimum reported cases in winter (ECDC, 2019a) (Figure 5).

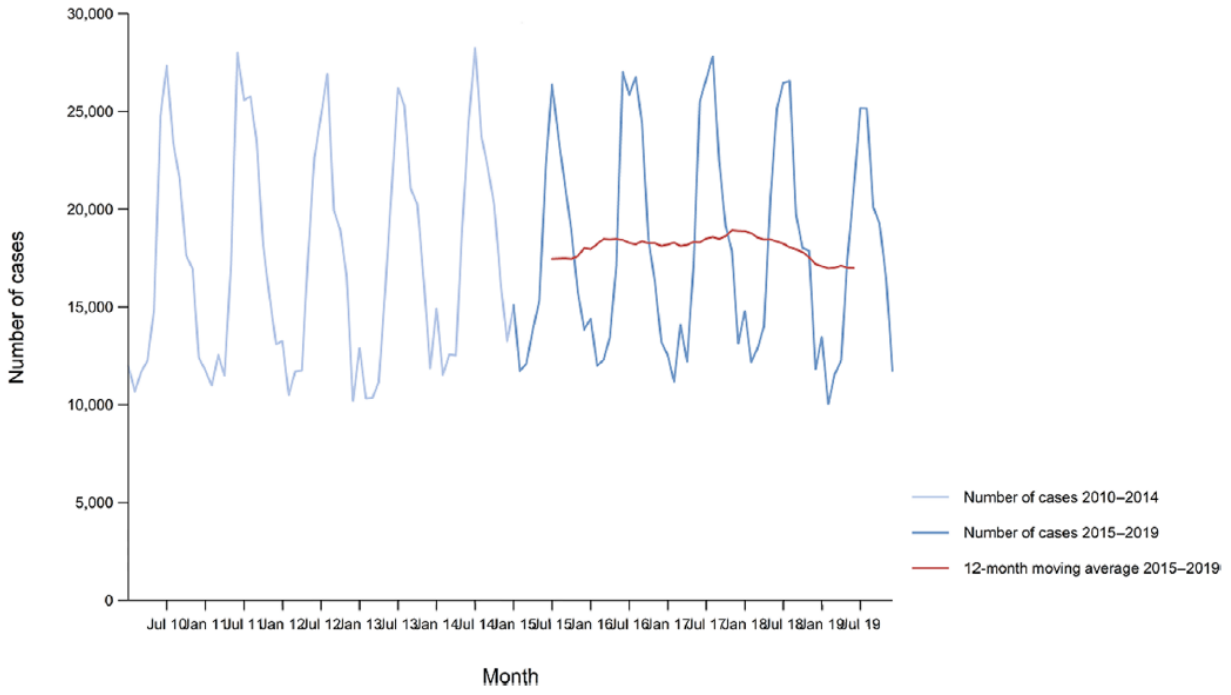

Figure 5: "Trend in reported confirmed human cases of campylobacteriosis in the EU/EEA, by month, 2015-2019" (extracted from (EFSA and ECDC, 2021a)).

New species of *Campylobacter* are regularly discovered, however human cases are dominated by two prominent species, including *C. jejuni* (83.1%) and, to a notably lesser extent, *C. coli* (10.8%). Other species including *C. lari*, *C. hyointestinalis*, or *C. upsaliensis* are rarely identified (6.1%) (EFSA and ECDC, 2021a). In 2019, according to the EU One Health zoonoses report, over 20,000 patients were hospitalized due to campylobacteriosis, representing the highest number of hospitalizations resulting from food-borne illnesses. This culminated in 47 deaths, leading to an EU case rare fatality rate of 0.03% (EFSA and ECDC, 2021a). More recent evidences in 2017 revealed that the disease was more prevalent in children under five years of age, and males compared to females (with an overall male-to-female ratio at 1.2:1) (Figure 6) (ECDC, 2019a).

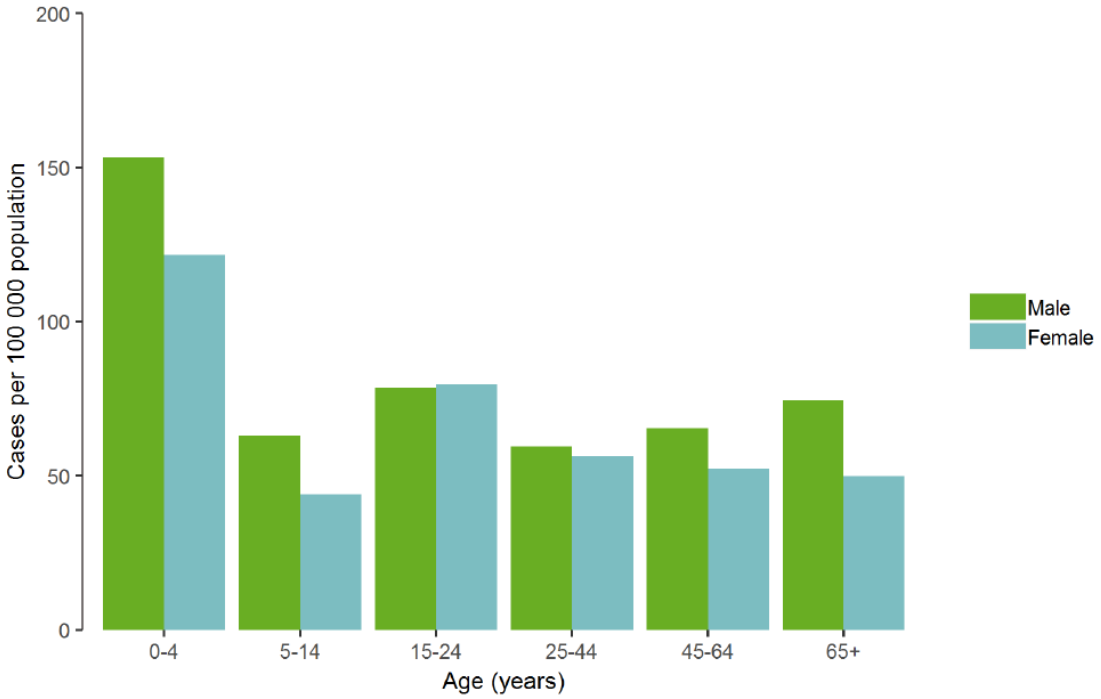

Figure 6: "Distribution of confirmed campylobacteriosis cases per 100,000 population by age and gender, EU/EEA, 2017" (extracted from (ECDC, 2019a)).

Campylobacteriosis has been found to be a significant health and economic burden worldwide. As such, it is estimated to contribute 2.4 billion euros cost-of-illness and 8.28 disability-adjusted life years (DALYs) per 100,000 population in Europe (Cassini et al., 2018; EFSA, 2021). Furthermore, it has become the third most prevalent death causing foodborne zoonosis in the EU, behind listeriosis and salmonellosis (Devleesschauwer et al., 2017; EFSA and ECDC, 2021a).

4.2. *Campylobacter* in animals and food

4.2.1. Wild and domestic birds

C. jejuni is asymptomatically carried into the digestive tract of wild and domestic birds. It is well adapted to the avian gut as it is thermotolerant and has an optimal growth temperature at 42°C, corresponding to the birds' body temperature. Reservoirs of *C. jejuni* include a wide variety of domestic and wild birds, the first being of particular importance to public health. Poultry corresponds to broiler chickens, hens, and turkeys, while in terms of wild bird carriage, a study highlighted that over 200 different bird species were colonized by *Campylobacter* species (Waldenström and Griekspoor, 2014).

4.2.2. Farm animals

Warm-blooded farm animals such as pigs, bovines, and sheep herd are well-known as secondary reservoirs for *Campylobacter*. *C. jejuni* is commonly isolated from healthy cattle and was confirmed as the predominant species in several studies (Hakkinen et al., 2007; Châtre et al., 2010; Mughini-Gras et al., 2012; Thépault et al., 2018b; Berthenet et al., 2019). In contrast, *C. coli* is more frequently isolated from sheep and pigs (Colles et al., 2003; Sproston et al., 2011).

4.2.3. Domestic pets and wild life

Campylobacter is also detected in the commensal microbiota of many other host species, including pets. Although most dogs and cats are healthy carriers, some of these animals could develop mild to moderate enteritis, the most prevalent species being *C. upsaliensis*, *C. helveticus*, and *C. jejuni* (Damborg et al., 2004; Acke, 2018; Thépault et al., 2020). In a major advance, Montgomery *et al.* (2018) surveyed *C. jejuni* infections in an American pet store chain where 142 puppies were positive, and 118 human cases were confirmed as being linked to these infected animals (Montgomery et al., 2018). *C. jejuni* is also widespread in nature and has been isolated from a wide range of sources, ranging from the common housefly to Antarctic penguins, reptiles, turtles, bats, and red kangaroos (Hald et al., 2007; Stirling et al., 2008; García-Peña et al., 2017; Gill et al., 2017; Ahasan et al., 2018; Hazeleger et al., 2018; Gilbert et al., 2019).

5. Transmission routes

The transmission of microorganisms can be divided in two types of routes: direct contact and indirect transmission (e.g., fomites, aerosol (airborne), oral (ingestion of contaminated food and water), and vector-borne). In the case of *Campylobacter*, transmission by contaminated food ingestion and, to a much lesser extent, direct contact with carrier animals are the main means of transmission. Consequently, campylobacteriosis is classified as zoonosis: the infection is transmitted from animals to humans and vice versa (WHO, 2020b).

5.1. Risk factors for humans

Recently, Mughini-Gras and colleagues (2021) led a risk factor analysis, which identifying factors associated with human infection related to *Campylobacter* strains attributable to specific sources. Undercooked chicken meat consumption was confirmed as the main risk factor for human campylobacteriosis (Figure 7) (Mughini-Gras et al., 2021). A case-control study confirmed that consuming chicken outside the home was the dominant risk factor for *C. jejuni* and *C. coli* in Luxembourg (Mossong et al., 2016). Other source-specific risk factors were linked to consumption of unpasteurized milk and undercooked or barbecued meat, barbecuing providing many opportunities for re- and cross-contamination (Davis et al., 2016; Jaakkonen et al., 2020; Mughini-Gras et al., 2021). Indeed, transfer of *Campylobacter* from naturally contaminated raw meat (e.g., chicken legs) to a cutting board occurred in 80% of the cases after 10 minutes of contact, consumers practices being directly linked to *Campylobacter* prevalence in kitchens (Fravalo et al., 2009; Guyard-Nicodème et al., 2013; Møretrø et al., 2021). These studies highlight the common risk factor linked to the consumption of cross-contaminated food (e.g., raw vegetables and fruits) (Verhoeff-Bakkenes et al., 2008, 2011).

Furthermore, owning puppies has also been reported to be a risk factor for human campylobacteriosis, especially for children, as their feces could be contaminated (e.g., the *C. jejuni* outbreak linked to puppy exposure in the USA between 2016 and 2018, with 118 persons infected) (Mughini-Gras et al., 2013; Montgomery et al., 2018; Thépault et al., 2020; Lemos et al., 2021). In other studies, direct contact with environmental sources have also been reported with recreational water (e.g., swimming) or professional exposure (e.g., humans in poultry farms and slaughterhouses) (Figure 7) (Mughini-Gras et al., 2013; Vegosen et al., 2015; Ravel et al., 2016; Duijster et al., 2019).

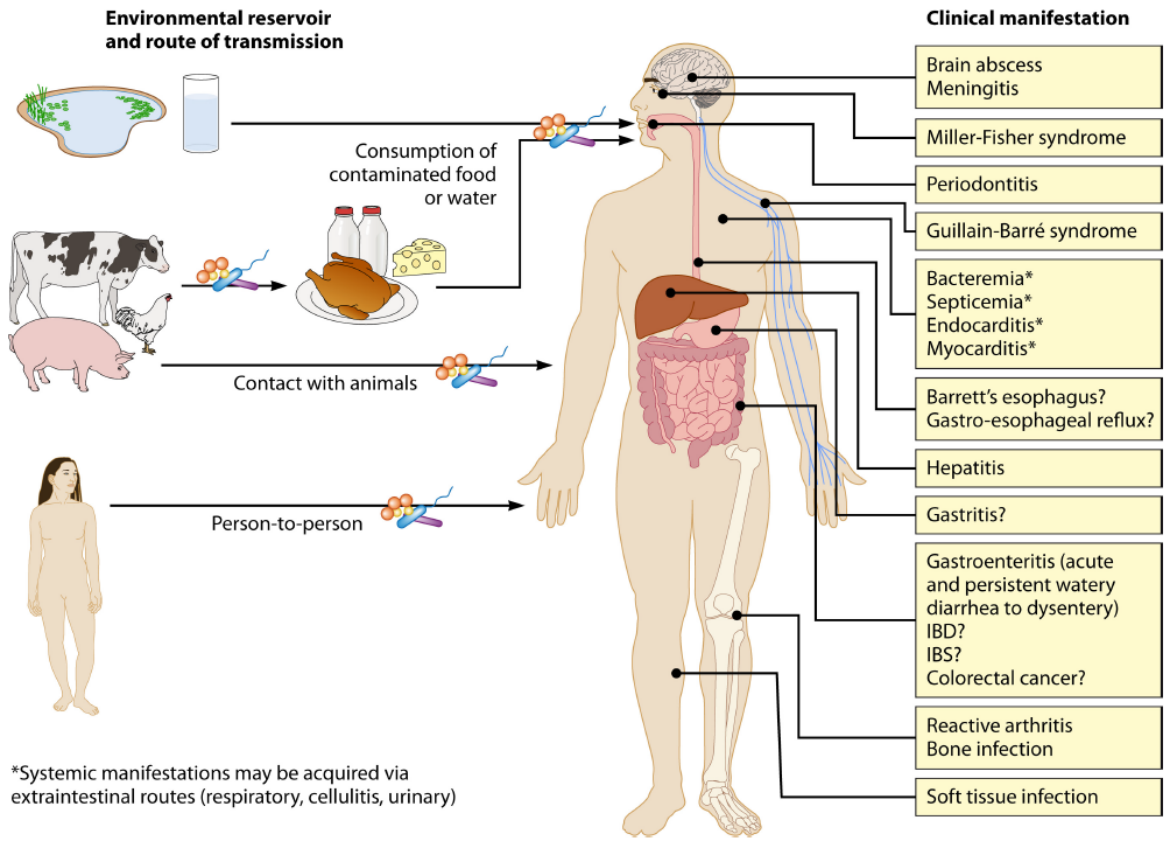

Figure 7: "Environmental reservoirs, routes of transmission, and clinical manifestations associated with *Campylobacter* species" (extracted from (Kaakoush et al., 2015)).

"IBD: inflammatory bowel diseases, IBS: irritable bowel syndrome. Question marks indicate conditions for which a role for *Campylobacter* is implicated but not certain." (extracted from (Kaakoush et al., 2015)).

5.2. Transmission routes to animal production

Campylobacter species were reported in abundance within poultry farms (i.e., soil, surface waters, wild and domestic animal feces), and they can be transmitted horizontally from the environment to poultry farms through vehicles resulting in contamination of animal food or drinking water (Figure 8) (Ellis-Iversen et al., 2012). In addition, other organisms, such as insects, are also important horizontal transmission routes into poultry houses, and fly screens were successfully used to significantly reduce *Campylobacter* introduction in broiler flocks (Hald et al., 2007; Newell et al., 2011).

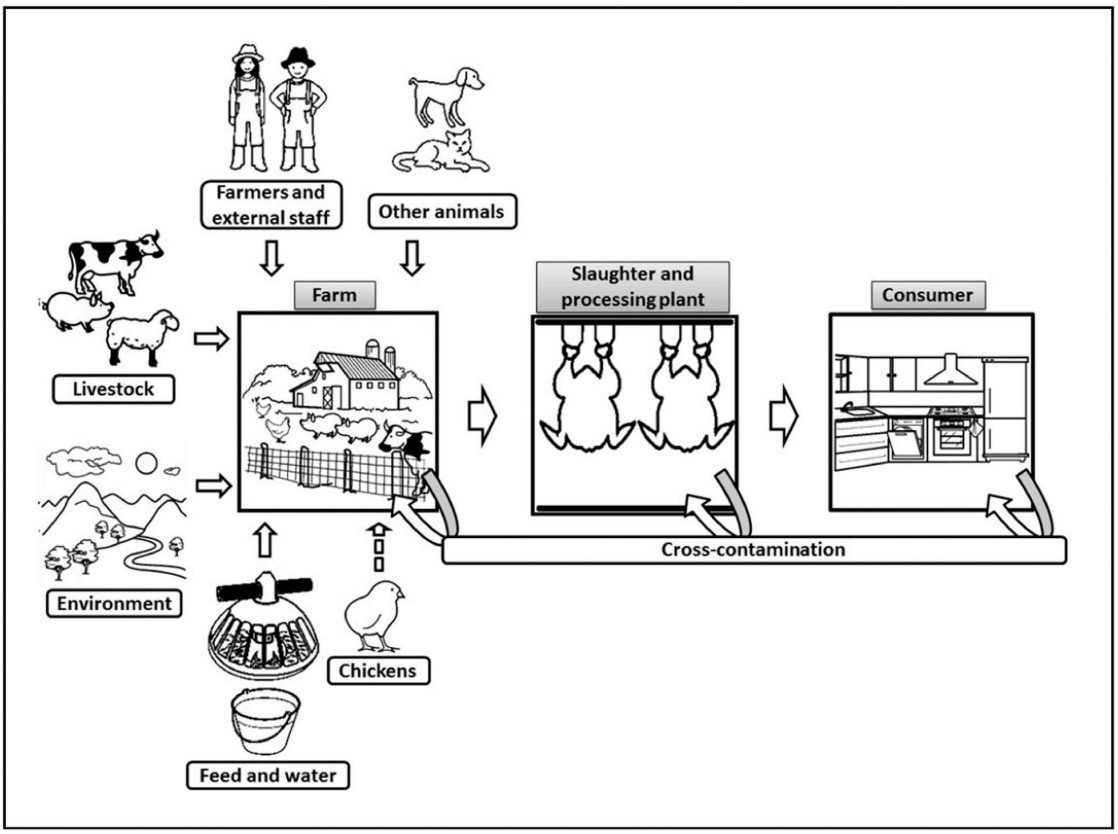

Figure 8: Contamination sources of *Campylobacter* through the poultry production (extracted from (Soro et al., 2020)).

Colonization in broiler chicks during rearing has been demonstrated to be a risk factor in the transmission of *C. jejuni* infection, prevalent during preharvest conditions. Furthermore, certain critical points for carcass contamination were also identified during plucking, evisceration, and final washing (Guerin et al., 2010). However, the potential of different intervention strategies was investigated in broiler production to prevent and reduce *C. jejuni* contamination level in the food chain (i.e., proper biosecurity measures including good hygiene practices, use of bacteriocins or probiotics, or phage therapy) (Soro et al., 2020). In addition, the EFSA Scientific Opinion on control options for *Campylobacter*, along the poultry meat production chain, estimates that "a public health risk reduction from the consumption of broiler meat of more than 50 % could be achieved if carcasses complied with a limit of 1 000 CFU.g^{-1}". Thus, this microbiological criterion has been applied since 2018 (Commission Regulation, 2017). The last review of control options for *Campylobacter* in broilers at primary production was carried out in 2020. In the updated model, an estimate of a 3-$\log_{10}$ reduction in broiler caecal concentrations could lead to a reduction of the relative EU risk of human campylobacteriosis attributable to broiler meat by 58% (EFSA, 2020).

6. Clinical features of *Campylobacter* infections

6.1. Campylobacteriosis disease

C. jejuni colonizes the lower gastrointestinal tract (GIT) with a low infectious dose ingested, with as few as 500 bacteria suffices as gastroenteritis causing (Robinson, 1981; Black et al., 1988). The incubation time can vary between 24 and 72 hours after ingestion, with a peak of illness lasting 24 to 48 hours and infection about a week (Man, 2011). *C. jejuni* causes acute and self-limiting enteritis, but it results in fatality among immunocompromised patients, elderly people, and very young children. The clinical signs are mainly digestive, the most prevalent being constant diarrhea, characterized by liquid and abundant stools (sometimes bloody). The common digestive tract symptoms are abdominal cramps, fever, headache, and, less frequently, vomiting (ANSES, 2011).

6.2. Late onset pathologies

6.2.1. Gastrointestinal manifestations

While gastroenteritis is the primary clinical illness, *C. jejuni* and other *Campylobacter* species are associated with the development of foodborne gastroenteritis-associated sequelae, such as inflammatory bowel diseases leading to chronic inflammatory conditions of the GIT (e.g., Crohn's disease and ulcerative colitis) (Hutchinson, 2009; Kalischuk and Buret, 2010; Scallan Walter et al., 2019; Peters et al., 2021). These lesions and chronic inflammations of the GIT result in dysregulations of the immune response. Furthermore, according to He *et al.* (2019), *C. jejuni* 81-176 strain can induce changes in gut microbial composition. Recently, a dysbiosis of the gut microbiota using the murine model has been shown to contribute to the development of colorectal cancer in presence of *C. jejuni* (He et al., 2019).

6.2.2. Extra gastrointestinal complications

C. jejuni has also been implicated in extra gastrointestinal clinical manifestations in a systemic reaction after an episode of enteritis or a post-infectious immune disorder. Bacteremia (i.e., presence of bacteria in the blood) is one of the most common extra gastrointestinal manifestations following *C. jejuni*, *C. coli* and *C. fetus* infections, for instance (Man, 2011). It occurs mainly in elderly and immunocompromised patients, who are also susceptible to septicemia (i.e., presence and multiplication of bacteria in the blood) (Nielsen et al., 2010). Another late-onset complication is the reactive arthritis, characterized by a

recurrent inflammatory arthritis of large joints (e.g., knees and ankles), conjunctivitis, and urethritis in men or cervicitis in women (García-Kutzbach et al., 2018). Its incidence resulting from campylobacteriosis ranged from 8 to 16% in adults and 0 to 6% in children (Pope et al., 2007; Ajene et al., 2013). Reactive arthritis symptoms can appear one month following the infection, complicating the identification of the *Campylobacter* infection source when the primary infection was not clearly identified in the patient.

These complications also include Guillain-Barré syndrome (GBS), a peripheral nervous system disease (Landry, 1859). This is a severe but rare complication (1.7 cases per 1,000) following a *C. jejuni* infection, described as a chronic and potentially fatal form of paralysis (Scallan Walter et al., 2020). Exposition to flu, Epstein Barr or Zika virus are also known to trigger GBS, making it difficult to identify the infection source (Tam et al., 2007; Vellozzi et al., 2014; Barbi et al., 2018). GBS causes progressive limb weakness and respiratory failure, leading to mechanical ventilation in about 25% of patients and an inability to walk after six months for 20% of cases (van Doorn et al., 2008). From 3 to 5% of patients die from respiratory muscles paralysis or cardiac arrest (Nachamkin, 2002; WHO, 2016). Molecular mimicry of *C. jejuni* lipooligosaccharides (LOS) at the human peripheral nerve gangliosides level is thought to induce an antibody cross-reaction (i.e., between both the bacteria and human nerve cells GM1 gangliosides) that leads to autoimmune disease resulting in neuron demyelination and degradation (Figure 9) (Yuki et al., 2004).

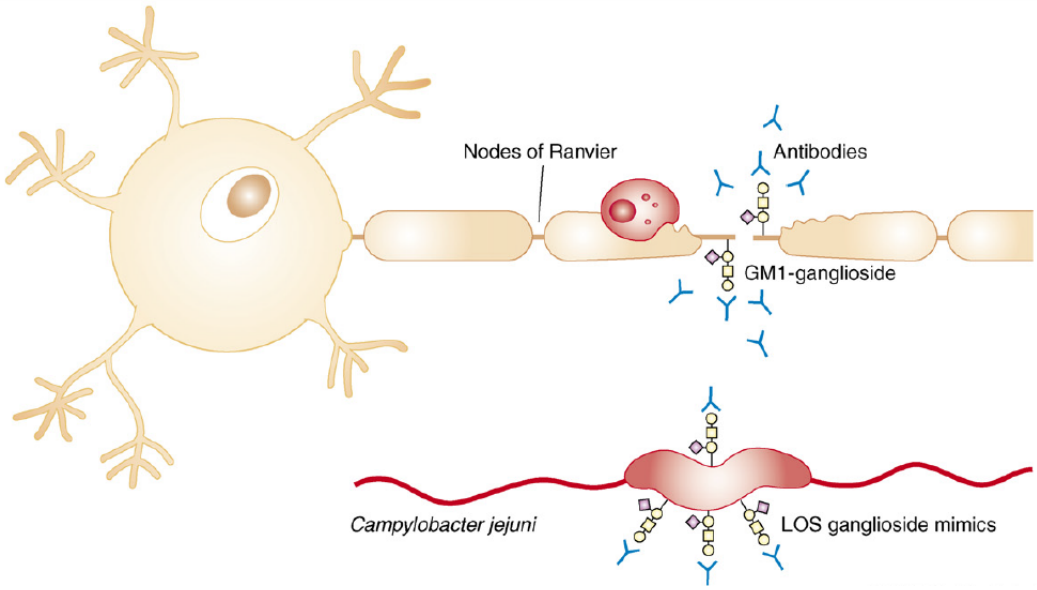

Figure 9: *C. jejuni* induced autoimmunity (extracted from (Guerry and Szymanski, 2008).

More precisely, in some *C. jejuni* strains, sialic acid which is incorporated into the outer-core regions of the LOSs, favors the mimic of human neuron gangliosides (Guerry et al., 2000; Huizinga et al., 2012). As a result, people at risk of developing GBS have a breakdown in self-tolerance and produce anti-ganglioside antibodies directed against *C. jejuni* LOSs, which in turn damage neurons in their peripheral nervous system (Bowes et al., 2002).

A clinical variant of GBS, the Miller Fisher syndrome (MFS) was also described as a complication associated to *C. jejuni* infection (Berlit and Rakicky, 1992). It has an autoimmune mechanism similar to that of GBS. It is characterized by ataxia (abnormal muscle coordination), ophthalmoparesis (paralysis of the eye muscles), and areflexia (absence of tendon reflexes) (National Institute of Neurological Disorders and Stroke, 2019). Similar to the GBS, molecular mimicry was also highlighted for this syndrome (Willison and O'Hanlon, 1999).

6.3. Pathogenicity and putative virulence factors

According to Shapiro-Ilan and colleagues (2005), pathogenicity is "the quality or state of being pathogenic, the potential ability to produce disease, whereas virulence is the disease-producing power of an organism, the degree of pathogenicity within a group or species" (Shapiro-Ilan et al., 2005). Concerning *Campylobacter*, relatively little is known about the mechanisms of pathogenicity, but they include four main stages: adhesion to intestinal cells, colonization of the digestive tract, invasion of targeted cells, and toxin production (Kreling et al., 2020). Furthermore, some virulence factors have been identified with a role in their capability to interact with, adhere to and invade host cells (Table 2) (Lugert et al., 2015).

Table 2: Putative virulence factors linked to the pathogenesis stages.

Pathogenesis stages	Putative virulence factors linked	References
Movement	Flagellum	(Lugert et al., 2015)
	T3SS	(Konkel et al., 2004)
Adhesion	CadF	(Konkel et al., 1997; Bolton, 2015; Lugert et al., 2015)
	PEB1, PEB3, PEB4	(Leon-Kempis et al., 2006; Min et al., 2009; Kale et al., 2011)
	CapA, JlpA	(Jin et al., 2001; Ashgar et al., 2007)
	MOMP	(Schröder and Moser, 1997; Wieczorek and Osek, 2013)
	Glycome	(Karlyshev et al., 2005; Habib et al., 2009; Thépault et al., 2018a)
Invasion	PEB1, PEB3, PEB4, CapA, JlpA	(Leon-Kempis et al., 2006; Min et al., 2009; Kale et al., 2011)
	Cia	(Bolton, 2015)
	CDT	(Pickett et al., 1996; Pickett and Whitehouse, 1999)

The motility of *C. jejuni* is known to be a crucial factor for invasion and colonization (Yao et al., 1994). The bacteria possesses a powerful polar flagellum which, in addition to its corkscrew shape, facilitates its high-speed movement through viscous substances (e.g., the mucus layer of the mucus membrane in the small intestine) and enables it to reach its target cells (Lugert et al., 2015). This flagellar system, also named type III secretion system (T3SS), is used as the export apparatus for immunogenic proteins CiaA and CiaB (Konkel et al., 2004). The flagellum contains a hook-basal body composed of several proteins (e.g., FliF, FliG, MotA/B, FlgE) and an extracellular filament structure formed by two flagellin proteins: FlaA and FlaB (Bolton, 2015). The movement of the bacteria is guided by chemotaxis, a mechanism by which motile bacteria detect and move to more favorable conditions, allowing *C. jejuni* to pinpoint primary colonization sites in the gut (Chang and Miller, 2006). When *C. jejuni* bacteria have reached the site, they have to adhere to the intestinal epithelial cells, which is essential for host colonization. Several bacterial surface proteins, called adhesins, mediate this attachment, such as the *Campylobacter* adhesion to fibronectin protein (CadF) (which specifically binds to cell membrane fibronectin) (Konkel et al., 1997; Bolton, 2015; Lugert et al., 2015). CadF has been shown to be linked to the caecal colonization *in vivo* in chicken and *in vitro* in human intestinal epithelial cells (Ziprin et al., 1999; Monteville et al., 2003). Furthermore, it has been suggested that CadF activates specific GTPases, facilitating the internalization of bacteria into host cells (Krause-Gruszczynska et al., 2007). Others proteins, such as PEB1, PEB3, PEB4, CapA, and JlpA, act as bacterial adhesins and are implicated in host (e.g., human or chicken) cell adhesion, invasion, and colonization (Jin et al., 2001; Leon-Kempis et al., 2006; Ashgar et al., 2007; Min et al., 2009; Kale et al.,

2011). The major outer membrane protein (MOMP) is also involved in adherence and antibiotic resistance (Schröder and Moser, 1997; Wieczorek and Osek, 2013).

After adhesion, the invasion process of *C. jejuni* is thought to be the most important step that causes damage to host cells (Lugert et al., 2015). Many proteins have been associated with this process, such as *Campylobacter* invasion antigen (Cia) proteins secreted by T3SS (Bolton, 2015). *Campylobacter* also produces several different cytotoxins, and the secretion of the cytolethal-distending toxin (CDT) has been linked with the invasion host cells (Pickett et al., 1996). This tripartite toxin is composed of three subunits encoded by the *cdtA*, *cdtB*, and *cdtC* genes. The CdtB proteins enter the nucleus, arrest the cell cycle G2/M phase by blocking the entry into mitosis and lead to cell death (Pickett and Whitehouse, 1999). The action of these CDT was also linked to the promotion of colorectal tumorigenesis (He et al., 2019).

Survival, adherence, and evasion of the host immune system could be supported by the *Campylobacter* glycome, composed of carbohydrate structures such as a polysaccharide capsule surrounding the surface of *C. jejuni* cells and sialylated LOS (Karlyshev et al., 2005). LOS have been associated with high invasive potential and are common to both human and poultry strains (Habib et al., 2009; Thépault et al., 2018a). However, the importance of the different putative virulence factors is far from clear; more studies on relevant clinical isolates are needed to get a comprehensive picture of the pathogenesis of *Campylobacter*, allowing the subsequent development of appropriate treatments. No receptor on human epithelial cells, neither protein of *Campylobacter* that could recognize these receptors were identified up to now. Consequently, it is not possible to define if the virulence factors identified belong to a direct or an indirect mechanism.

6.4. Treatment and antibiotic resistance

6.4.1. Treatment

Campylobacteriosis is usually self-limiting and requires no therapeutic intervention other than alleviation of symptoms and intake of supplements to maintain hydration and electrolyte balance. However, immunocompromised patients, those who develop severe or persistent symptoms, as well as those with extra gastrointestinal disorders should receive antibiotic treatment (Centers for Disease Control and Prevention, 2019b). Laboratory tests are performed to determine the antibiotic susceptibility of the strains in order to ensure appropriate and timely treatment when necessary. The first-line treatment of human campylobacteriosis includes macrolides (mostly erythromycin), which mechanism of action

resolves around their ability to bind the 50S subunit of the ribosome inhibiting bacterial RNA-dependent protein synthesis (Patel and Hashmi, 2021). Gentamicin and erythromycin are both used due to their lower resistance rate; erythromycin and gentamicin resistances to *C. jejuni* isolates are 1.5% and 0.3%, respectively (Centers for Disease Control and Prevention, 2019b; EFSA and ECDC, 2021b). Quinolones are not generally appropriated to alleviate symptoms, as *C. jejuni* strains show increasing resistance to fluoroquinolone antibiotics worldwide, reducing the efficacy of the treatment. However, it stays one of the first-line treatment in certain countries, such as Nordics countries, as the resistance level is practically non-existent (EFSA and ECDC, 2021b).

6.4.2. Antibiotic resistance

Antibiotics are medications that kill (bactericide) or slow down bacterial growth (bacteriostatic). Therefore, antibiotic resistance can be defined as a loss of susceptibility of bacteria to an antibiotic, making it ineffective against the infection and representing a clinical issue with the risk of treatment failure (Centers for Disease Control and Prevention, 2020). AMR is mainly caused by the overuse and misuse of antibiotics in humans and in animal production, including inappropriate use in healthcare or excessive use in agriculture (Sutherland and Barber, 2017). These drugs were initially used at low doses in animal feeds as a growth promoter, which allowed the product quality improvement, with a lower fat percentage and higher protein content in the meat. In the 1980s, an increase in fluoroquinolone (FQ) resistance was observed in human cases, suggesting that the use of this antibiotic in poultry was the source (Endtz et al., 1990). This hypothesis has been reinforced by the results of more recent studies showing the isolation of quinolone-resistant *Campylobacter* strains in large numbers from commercial chicken flocks, following antibiotic treatment at different time points of the poultry food chain (Griggs et al., 2005; Wieczorek et al., 2018). Interestingly, data have highlighted a low level of resistance to FQ in humans in Australia, probably because this antibiotic has never been approved for use in animals (Cheng et al., 2012). FQ were categorized as critically important drugs for human medicine, and surveillance programs to monitor their use in the veterinary field were implemented in the EU in 2011. They are now authorized only for therapeutic purposes in this sector (European Medicines Agency, 2013). With the implementation of restrictive use, global antimicrobial sales decreased by 43.2% between 2011 and 2020 in livestock, according to the significant decrease in antibiotic use (European Medicines Agency, 2021).

Despite the restrained use of antibiotics, *C. jejuni* strains are still resistant to fluoroquinolones in many areas of the world. This resistance is mainly due to a point mutation in the *gyrA* gene, coding for the gyrase

A subunit. The modification of this site results in an amino acid substitution in the quinolone resistance determining region. The most frequent mutation is the C257T alteration in the gene, leading to the Thr86Ile substitution. The fluoroquinolone binding sites are altered, and the antibiotic affinity decreases (i.e., can no longer bind to its target) (Wieczorek and Osek, 2013). The spread of this mutation led to a ciprofloxacin resistance level of 61.5% in *C. jejuni* human clinical strains in 2019 in the EU, followed by a tetracycline resistance in 47.2% of *C. jejuni* strains. This second resistance is mainly caused by the production of Tet(O) ribosomal protection proteins (Wieczorek and Osek, 2013; EFSA and ECDC, 2021b). Antibiotic resistance has increasingly become a problem, and the case of *Campylobacter* is far from being an exception. In 2019, in Europe, 69% and 77% of *C. jejuni* and *C. coli* human isolates, respectively, were resistant to one or two antimicrobial classes. For instance, 40.3% of *C. jejuni* isolates showed a combined resistance to ciprofloxacin and tetracycline (EFSA and ECDC, 2021b). According to an estimate, about 700,000 people die every year of bacterial infections due to multi-drug resistance (Sutherland and Barber, 2017). The team led by Jim O'Neill also estimated that the number of deaths due to AMR could reach 10 million people each year by 2050 if no action is taken soon (Figure 10) (O'Neill, 2016).

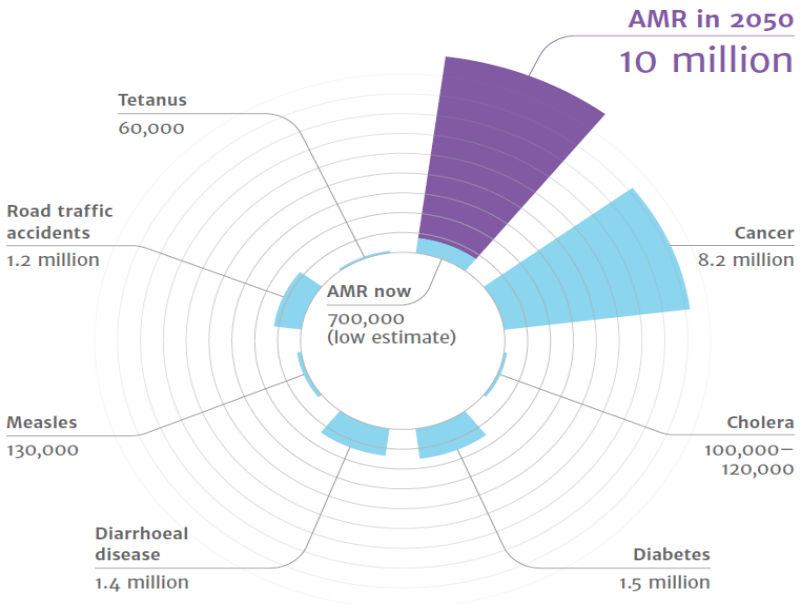

Figure 10: Deaths due to antimicrobial resistance and other diseases each year, and the projection by 2050 (extracted from (O'Neill, 2016)).

6.5. Vaccine development

Human campylobacteriosis represents a significant public health issue, and poultry has been identified as an important source of human infections. Furthermore, implementing adequate controls to reduce clinical disease burden is considered a priority with the rise of antibiotic resistance. Therefore, the management of *Campylobacter* colonization in poultry is crucial: a vaccination campaign in chickens would not only reduce infection levels in broilers but would also reduce transmission to humans and allow the elimination of many post-harvest procedures (de Zoete et al., 2007). These objectives have been achieved in the context of *Salmonella* infections: its prevalence was lower in broilers after the vaccination program and disposal of carriers in breeding flocks (Dórea et al., 2010). Several trials have been carried out to design an effective vaccination for poultry against *Campylobacter*, but so far, no efficient vaccine has met success (Mauri et al., 2021; Pumtang-On et al., 2021).

Several assays were also performed to study the development of a vaccine for humans. Despite its global importance, the development of a vaccine against *Campylobacter* has been delayed by its antigenic diversity, a poor understanding of its pathogenesis, and the lack of small animal models. Indeed, ideally, *in vivo* models should use small laboratory animals that are free of specific pathogens, genetically similar, with known immunity and readily available. The animal model should also develop the disease in a manner similar to humans. These criteria are required for the validation of an *in vivo* model leading to reproducible and verifiable results (Newell, 2001). The disease has been strongly represented using free-ranging rhesus macaques as experimental models, replicating human-like campylobacteriosis, although some ethical questions are addressed about the use of these animal models (Quintel et al., 2020). Many trials were performed with other animals; however, it has been challenging to establish *C. jejuni* infections due to its inability to cause disease in many common animals. For example, the chick model, being inexpensive and extensively used in *C. jejuni* studies, does not allow the reproduction of typical disease patterns as seen in humans. However, some works have successfully developed and utilized relevant mouse models (Bereswill et al., 2011; Stahl et al., 2014, 2017). This has led to significant advances in vaccine development for humans, even though potential vaccine candidates are still being studied without success to date (Poly et al., 2018). The pandemic SARS-COV2 crisis has recently pushed towards recent multi-omics approaches, which lead to reveal potential core vaccine targets against *C. jejuni* (Cao et al., 2021).

7. Surveillance systems and foodborne outbreaks

Campylobacteriosis reporting is different for each country based on differing surveillance systems. The notification of the disease has been mandatory for most of the European Union Member States (EU MS), including Luxembourg since 2020 (Gouvernement du Grand-Duché de Luxembourg, 2019). Only five EU MS have a national surveillance system where notifications are voluntary-based, including France (EFSA and ECDC, 2021a). Consequently, data comparison between countries should be carefully interpreted, since the reported cases of *C. jejuni* and *C. coli* human infections are likely to represent only the tip of the iceberg due to underestimation.

In Luxembourg, integrated surveillance of *C. jejuni* genetic profiles was implemented at the national level since 2005 through an initial research project EPIFOOD (FNR/03/07/08, CORE project-2005-2009). As a result, isolates from patients, food, animals, and environmental sources have been routinely collected over the past 15 years. The full national coverage of the surveillance system could explain the very high notification rate observed in Luxembourg compared to other EU countries (103.8 cases per 100,000 population *vs*. 64.8 cases per 100,000 at EU level in 2018) (EFSA and ECDC, 2019). However, since 2019, a surveillance artifact has been caused by the notification rate linked to the implementation of non-culture-based methods in biological medical laboratories (i.e., detection by PCR, and isolates no more collected), leading to a seemingly lower incidence.

Although monitoring systems differ from country to country, reporting foodborne campylobacteriosis disease outbreaks in humans is mandatory according to the Zoonoses Directive 2003/99/EC (European Parliament, 2013). In 2019, *Campylobacter* was the third causative agent for food-borne outbreaks in the EU, with 319 outbreaks declared to EFSA, involving over 1,200 cases of illness, 125 hospitalizations, and no deaths. Most of them were reported without speciation information; however, *C. jejuni* and *C. coli* were identified in respectively 72 and 7 outbreaks. According to the EFSA guidelines, 18 outbreaks were classified with strong-evidence and 301 with weak-evidence (Figure 11) (EFSA, 2014).

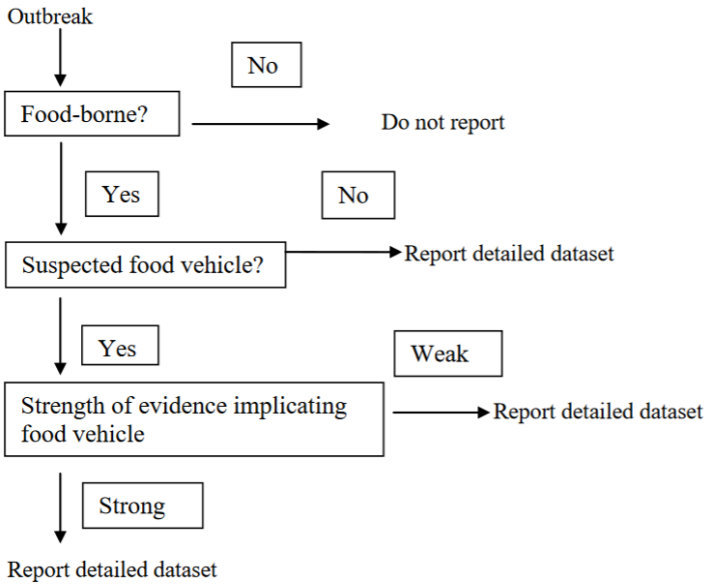

Figure 11: Scheme for reporting of food-borne (excluding water-borne) outbreaks to the updated EU Food-borne Outbreak reporting system (updated EU-FORS) (extracted from (EFSA, 2014)).

8. Diagnostic tools and techniques to monitor *C. jejuni*

8.1. Detection methods

Campylobacter spp. are zoonotic, and most of the cattle and poultry carry the bacterium asymptomatically. Poultry is the primary source/reservoir of *Campylobacte*r human infections. Other animal products, such as red meat, unpasteurized raw milk, fresh produces, and contaminated waters may harbor the pathogen (EFSA and ECDC, 2021a). Thus, the accurate and rapid detection of *Campylobacter* spp. is critical for monitoring the risk exposure throughout the food production as well as for reporting campylobacteriosis incidence. Several methods have been developed to detect this pathogen, either for clinical diagnosis or for screening in food or the environment.

8.1.1. Laboratory clinical diagnosis

Different diagnostic methods are available for *C. jejuni* infection detection, using culture-dependent and/or culture-independent methodologies, such as stool cultures and PCR-based methods. For clinical diagnosis, stool cultures are usually performed with the seeding of the stool samples on a selective growth medium followed by an incubation at 42°C in microaerophilic conditions. This method is well adapted for detecting *C. jejuni*, which are thermotolerant (Costa and Iraola, 2019).

Nowadays, many clinical laboratories complete their detection analyses using the MALDI-TOF MS (matrix-assisted laser desorption ionization - time of flight mass spectrometry) to identify and confirm the isolates to the species level. Its use was scientifically validated (International Organization for Standardization, 2019a; Akimowicz and Bucka-Kolendo, 2020). It ionizes samples (e.g., bacterial colony) into charged molecules, the ratio of their mass-to-charge (m/z) being measured and resulting in a mass spectrum. This method has been validated to rapidly identify seven *Campylobacter* species (*C. jejuni*, *C. coli*, *C. lari*, *C. fetus*, *C. upsaliensis*, *C. helveticus*, and *C. sputorum*). However, other species are more difficult to detect, possibly due to the absence or lack of reference spectra in the database (Hsieh et al., 2018).

The 16S rRNA method was also used for rapid detection and identification. Still, it cannot differentiate very closely related species, as there is insufficient sequence variation in the 16S rRNA gene (e.g., *C. jejuni* and *C. coli*, whose sequences shared over 98% sequence similarity) (Burnett et al., 2002). Many PCR-based methods were developed to detect more uncommon species and the use of multiplex PCR is an advantage as it allows the quick detection of a wide range of different enteropathogens (Park et al., 2011).

Culture-independent diagnostic tests (CIDTs) (i.e., techniques that can identify the general type of bacteria causing illness within hours, without having to culture, or grow the bacteria in a laboratory) are also increasingly used in routine (e.g., from 13% in 2012-2014 to 38% of *Campylobacter* diarrheal illnesses diagnosed only by CIDTs in 2018 in FoodNet sites) (Centers for Disease Control and Prevention, 2019a). Although they may enhance detection sensitivity, they may affect public health surveillance, as they do not provide isolates for further identification, typing (e.g., whole-genome sequencing), or antibiotic susceptibility testing. These latest characterization tests give detailed phenotypic or genomic profiles of isolates and are needed to classify strains to monitor trends and rapidly detect human clusters. Therefore, the increased use of CIDTs will likely result in a significant burden on public health laboratories in terms of surveillance (Iwamoto et al., 2015; Centers for Disease Control and Prevention, 2019a).

8.1.2. Detection in food and environment

Identifying *Campylobacter* in food is governed by several International Organization for Standardization (ISO) standards, depending on the method chosen. Culture-dependent methodologies are employed, such as the horizontal one for the detection by enrichment or direct plating of *C. jejuni* (International Organization for Standardization, 2017a, 2017b). These ISO standards apply to products intended for human consumption and animal feed, environmental samples taken in the food production and handling

sectors, and samples at the primary production stage such as animal feces, dust, and surface samples (International Organization for Standardization, 2017a). Depending on the sample type and the test's purpose, three research procedures can be used: procedures A and B are used for testing by enrichment in samples containing a low number of *Campylobacter*, with low or high additional flora, respectively. Procedure C is performed for testing by direct plating in samples containing large numbers of *Campylobacter*. Procedures A and B allow the analyses on the same sample quantity: 10 g or 10 mL, while procedure C is applied after swab or loop sampling (Figure 12) (International Organization for Standardization, 2017a, 2017b). Monitoring of *Campylobacter* on broiler carcasses is performed following the Regulation (EC) No 2073/2005. It assesses the counts above 1,000 $CFU.g^{-1}$ of *Campylobacter* on neck skins from broiler carcasses after chilling, considering a set of 50 pooled samples derived from 10 consecutive sampling sessions (Commission Regulation, 2017).

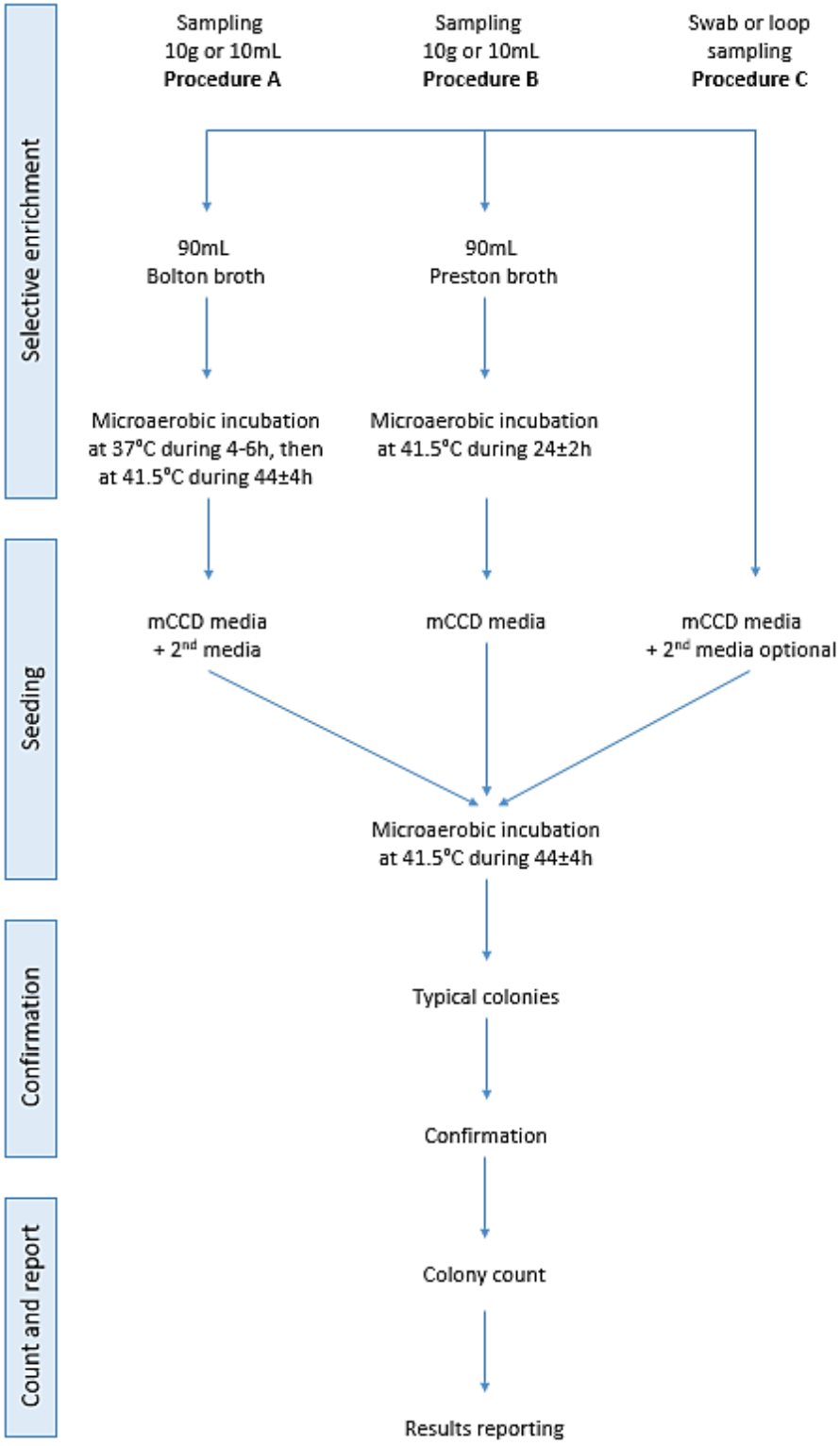

Figure 12: Schematic representation of the procedures for the detection of *Campylobacter* in the food chain (adapted from (International Organization for Standardization, 2017a, 2017b)).

A new and innovative method of gene amplification called loop-mediated isothermal amplification (LAMP), was recently developed to detect *Campylobacter* spp. in poultry samples with and without enrichment (Romero and Cook, 2018; Babu et al., 2020). It does not require bacterial culture or DNA purification and combines speed (i.e., samples are amplified at a fixed temperature within one hour), ease of use, and high specificity (Notomi et al., 2015). However, a recent study demonstrated that greater efforts are still needed to improve the sensitivity of this promising approach (Llarena et al., 2022). Finally, concerning environmental detection, and more specifically in water, the method and enumeration protocol of thermotolerant *Campylobacter* spp. are described in the ISO standard 17795 (International Organization for Standardization, 2019b). Another protocol, based on a filtration technique, is also used for water (or stool) samples: a sterile membrane filter, with a pore size of 0.45 or 0.60 µm (allowing only the migration of *Campylobacter*) is placed on the surface of the selective agar medium (Kulkarni et al., 2002; Tilmanne et al., 2019).

8.2. Typing methods

In epidemiology, the main purpose of molecular characterization of strains is to compare them for ruling on/out their genetic relationship. The related applications include: the detection of human clusters, the investigation and identification of potential outbreaks sources, the tracing back of transmission routes, and the monitoring of clinical studies. It also helps and provides information on virulence and antibiotic resistance to develop more adapted medical treatments. For *Campylobacter* typing, phenotypic and genotypic methods have been developed.

8.2.1. Phenotypic typing tools

Within these methods, the typing of the bacterial strains is based on phenotypical characters such as biochemical reactions and environmental tolerance (Eberle and Kiess, 2012). The biotyping methods characterize the metabolic activities of *Campylobacter* spp., and these results can be completed with the use of serotyping methods. Indeed, before robust genotyping methods were developed, serotyping was widely used to characterize the *Campylobacter* population. In the 1980s, two main serotyping schemes, the Penner and the Lior schemes, were used. They were based on heat-stable antigens and heat-labile antigens, respectively (Penner and Hennessy, 1980; Lior et al., 1982). These serotyping methods were expensive and time-consuming, with a high number of untypable isolates and a substantial number of

cross-reactions. Then, laboratories implemented one of the first methods used for genetic and epidemiological studies: the multi locus enzyme electrophoresis (MLEE). This is a non-DNA method based on enzymes polymorphism and gel electrophoresis. The migration of the proteins during electrophoresis is determined by the amino acid sequence of the peptide. Hence, the visualization of the banding pattern allows the identification of strains. However, many nucleotide substitutions did not result in amino acid changes (silent mutations), leading to a low-resolution power to assess microevolutionary changes or investigate outbreak clusters (Stanley et al., 2004).

8.2.2. Molecular typing tools

The expanding use of genotyping methods has enabled laboratories to conduct investigations during epidemiological outbreaks, laboratory contamination, or recurrent infection phenomenon. Genome analysis of viruses or bacteria allows the identification of variants, genes, and gene functions. It also reveals virulence factors and drugs resistances. Epidemiologists and regulatory agencies (e.g., ECDC or CDC) use it for surveillance of infectious diseases or monitoring of nosocomial events, but it is also useful in the research field of population genetics. Indeed, genomics provide new insights with high resolution on relatedness between strains according epidemiological data collected during sampling. For this purpose, the genomic profiles generated are compared with defined rules for interpreting if two isolates potentially derive from a same ancestor. Differences between two isolates could be expressed either in number of single nucleotide polymorphisms (SNPs) or difference in alleles (AD), and, to be clustered, a defined threshold or cut-off value has been generally validated through a collection of related isolates originated from diverse outbreaks. Furthermore, a common nomenclature that allows laboratory-independent identification and classification and exchange of data is essential to interpret and report results. Threshold and nomenclature are both method-dependent, as a wide variety of methods have been developed using nucleic acid-based technologies. The prevalent methods developed for *Campylobacter* typing include molecular fingerprinting, nucleic acid amplification tests, and sequence-based typing.

8.2.2.1. Before next-generation sequencing (NGS)

In 1984, Schwartz and Cantor devised a way to separate large molecules of DNA, leading to the birth of the pulsed field gradient gel electrophoresis (PFGE) (Schwartz and Cantor, 1984). For *Campylobacter*, this

method is based on restriction digestion of its chromosome, using rare-cutting restriction enzymes (e.g., *Knp*I or *Sma*I), into a small number of large fragments ranging from approximately 50 to 800 kb. First, their separation was performed in an agarose gel matrix utilizing the influence of two electric fields alternating their polarity. Then, the gel was stained and observed with ultraviolet (Ribot et al., 2001). This technique was widely used in molecular typing studies. It can be applied as a subtyping method and a suitable tool for epidemiological surveillance in a short timeframe (e.g., investigation of an outbreak or evolution of the diversity and contamination all along the process at slaughterhouse). However, there are some limitations: it is time-consuming (turnaround time of 24 to 72 hours), and it is not a well-adapted tool for long-term monitoring (Wassenaar and Newell, 2000). Initially, there was also a poor scalability and additional efforts were needed to achieve sufficient reproducibility to compare patterns across multiple gels, particularly among different laboratories. The development of international molecular subtyping networks, such as PulseNet, has facilitated the implementation of adequate normalizations of patterns and accurate fragment sizes estimates (Swaminathan et al., 2006). Digestion with one enzyme is sufficient in many cases to show differences between *Campylobacter* isolates but seems to be insufficient for establishing more accuracy of the relatedness between them. For this latest purpose, the combination of at least two or three enzymes can be used in separated experiments (Figure 13) (On et al., 1998). Furthermore, PFGE complex groups are defined thanks to a similarity cutoff value of 90% (de Boer et al., 2000).

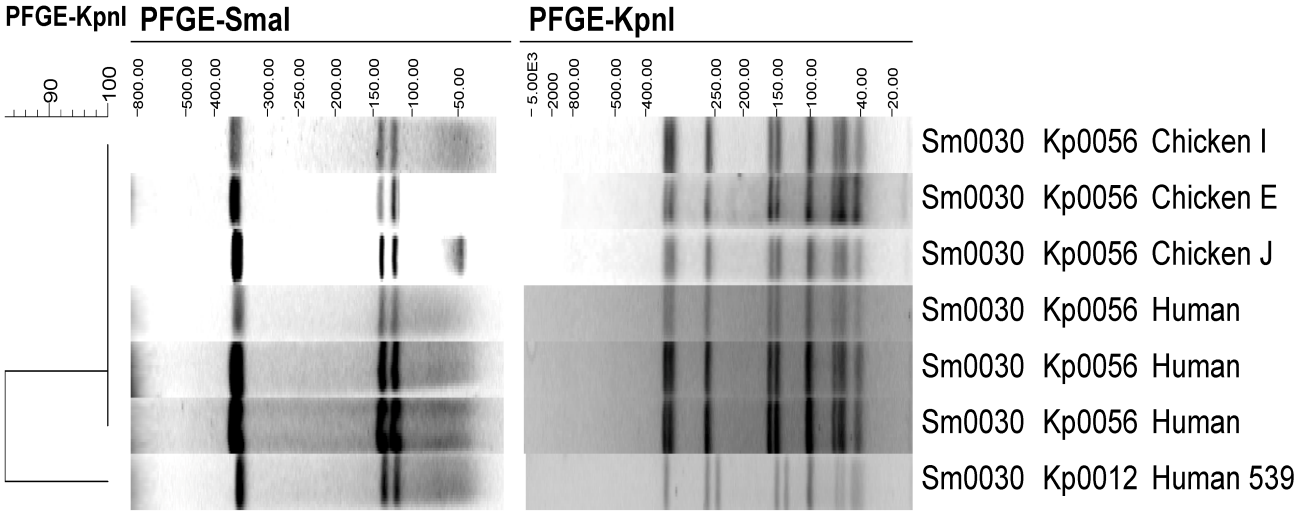

Figure 13: Clonal relationship between *Campylobacter jejuni* human and chicken isolates (extracted from (Devane et al., 2013)).

Then, in the late 90s, the amplified fragment length polymorphism (AFLP) was developed. It is hinged on whole-genome polymorphism and has been used for inter- and intraspecific differentiation of *Campylobacter* spp. It is highly discriminatory and advantageous as only one protocol can achieve the same resolution as PFGE. A cut-off value of 90% of similarity was implemented to determine if two isolates are related or not (de Boer et al., 2000). However, interpretation of typing results is difficult when isolates have similar profiles, as no common nomenclature and database are available. Furthermore, the method is costly and time-consuming (Duim et al., 1999; Kokotovic and On, 1999).

In the early 2000s, polymerase chain reaction (PCR) amplifications were developed. This method, followed by DNA sequencing of its products, was initially used for single locus genotyping methods such as *fla*A typing. The flagellin is an valuable target for discriminating among *C. jejuni* isolates, providing a high level of discrimination even when used alone (Corcoran et al., 2006). However, *fla*A alleles lack stability due to recombination and intraspecies transfer (Dingle et al., 2005). This typing method is unsuitable for long-term investigation but appropriate for distinguishing closely related strains when combined with more conserved genes (Dingle et al., 2008).

Multilocus sequence typing (MLST) was first described for *C. jejuni* and *C. coli*, in 1998 (Maiden et al., 1998). This gene-by-gene approach is based on the DNA sequencing of seven housekeeping genes (*aspA*, *glnA*, *gltA*, *glyA*, *pgm*, *uncA*, *tkt*). Each sequence variant represents a distinct allelic type, and combining the seven allele results in a sequence type (ST). Then, an algorithm called BURST was used to classify the MLST data set into groups of related isolated and clonal complexes (CC) (Dingle et al., 2001). Thus, the STs were grouped into CC, and CC members were defined as isolates with an ST that shared identical alleles at four or more loci (Dingle et al., 2001). MLST has become one of the most extensively used molecular typing methods for *Campylobacter* spp. It was the gold standard during the last decade, being used for many studies: strain diversity within hosts, possible host associations, evolution, pathogenicity, and epidemiology, as it is suitable for long-term studies (Sheppard et al., 2009; Strachan et al., 2009; Jolley and Maiden, 2014). To achieve higher discriminatory power for longitudinal studies, MLST was combined with additional targeted loci that are under selective pressure, such as the antigenic genes *fla*A and *fla*B, the gene *por*A encoding an outer membrane protein, and the *gyr*A encoding the subunit A of the gyrase, thus setting up the extended MLST (Clark et al., 2007; Dingle et al., 2008; Ragimbeau et al., 2014). The MLST – *gyr*A allele pairing could also increase the resolution of source attribution, while MLST – *por*A/*fla*A

alleles combination could be suitable for detecting temporal clusters of human cases (Ragimbeau et al., 2014).

Another locus based- sequencing method focuses on LOS typing. They are integral components of the *Campylobacter* cell membrane with a structure of core oligosaccharides forming inner and outer core regions and a lipid A moiety. LOS analysis was initially performed to classify isolates according to the risk of developing GBS later on. However, the gene content of the LOS core biosynthesis cluster exhibits significant variation, LOS locus being considered as a hot spot for genetic exchange and rearrangements (Parker et al., 2005). Analysis of the LOS biosynthesis genes by PCR assays could therefore be used for typing *C. jejuni*, with the implementation of a classification system with 23 classes (A – W) and four groups (1 – 4) for the *C. jejuni* LOS region. Although some studies have shown concordance between specific STs and LOS classes, no definite correlation has been established. Furthermore, this method does not allow to determine the genetic distance between two isolates, and genomic-based classification of the LOS region remains incomplete for now (Hameed et al., 2020).

The MLST method has been the gold standard in epidemiology and is still a reference data that is now deduced from the whole genome sequencing (WGS); however, the NGS era is revolutionizing this field by providing more information.

8.2.2.2. Genotyping through next-generation sequencing

In the 1970s, the Sanger method of DNA sequencing was developed to determine the nucleotide sequence of short fragments of 600 pb on average (Sanger et al., 1977). Then, this technique was optimized and combined with computational methods, setting up the shotgun sequencing technology (i.e., first-generation sequencing) which enables the whole-genome sequence of bacteria in one run (WGS). The first completed bacterial genome sequenced was *Haemophilus influenza* (Fleischmann et al., 1995). A decade later, the next-generation sequencing (NGS) methods were introduced and platforms were developed, such as Illumina, PacBio, and IonTorrent platforms.

NGS can be divided into two categories: the second-generation (SG) (e.g., Illumina, short reads) and the third-generation (TG) (e.g., Nanopore, long-reads) sequencing. In general, the SG produces shorter reads with a lower error rate and at a lower cost than TG, and it allows for a larger throughput of samples. However, the TG has several advantages, such as the real-time sequencing and the longer reads, making easier *de novo* assembly. It also provides better accuracy of repetitive regions sequence and easier

detection of mutations (Metzker, 2010; Loman et al., 2012). The workflow of the two methods involves DNA extraction and library preparation steps, followed by sequencing but not based on the same principle.

The SG sequencing mainly use sequencing by synthesis, in which DNA polymerases are used to incorporate nucleotides onto existing template strands, followed by detection of signals generated by the added nucleotides. For instance, Illumina technology is based on a particular sequencing-by-synthesis method: the pyrosequencing. Each cycle of the sequencing corresponds to a PCR cycle, and all the DNA fragments act as templates in the presence of a mixture of four fluorescence-labeled deoxynucleotides triphosphates (dNTPs). During each cycle of the PCR, there is an incorporation of one nucleotide, producing a light emission. Reading is performed to identify which dNTP was incorporated, and then the fluorophore is removed, allowing the start of a new cycle. Each recorded light signal is subsequently computationally transformed into the sequence reads (Figure 14) (www.illumina.com).

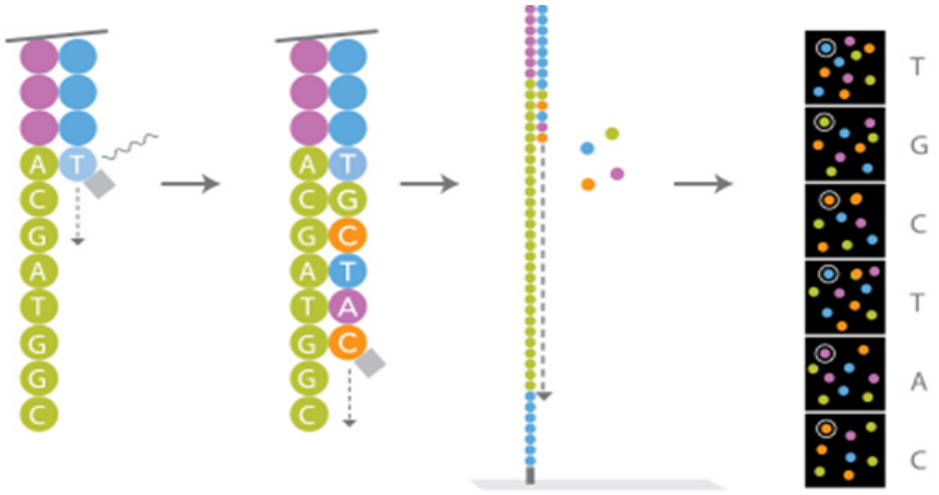

Figure 14: Sequencing-by-synthesis and sequence reading with Illumina technology (extracted from www.illumina.com).

Differently, the TG sequencing is based on a direct approach without DNA synthesizing and does not require DNA shearing nor PCR amplification. For instance, Oxford Nanopore Technologies is a real-time sequencing technique detecting disruptions of an electrical current in a membrane (i.e., a DNA molecule to be sequenced passes through a protein nanopore lodged into a high electrical resistance membrane and causes these disruptions). These signals are then used to distinguish which nucleotide passed through, and the DNA sequence is determined (Figure 15).

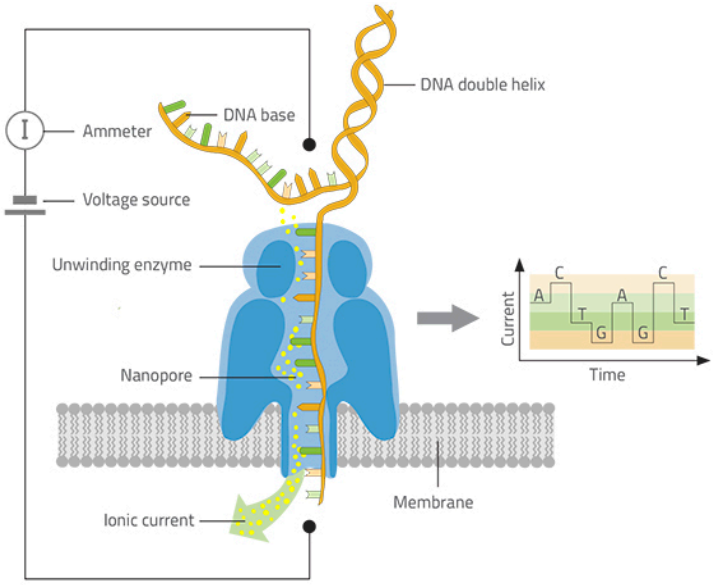

Figure 15: "Illustration of the Oxford Nanopore technologies working principle. A strand of DNA is passed through a nanopore protein, and an ionic current is measured and translated into the DNA sequence" (©Kerstin Göpfrich, www.scienceinschool.org).

The analysis of the raw WGS data is also different from one technique to another. At the end of the Illumina sequencing, data generated, called raw reads, are stored as FASTQ files. Illumina technology allows to sequence in one direction only (single-end reads) or in two directions (paired-end reads). Thus, two FASTQ files are generated for one sample in the case of paired-end sequencing. Then, the raw reads are trimmed, i.e., low-quality reads are removed (e.g., low coverage). The reads are then assembled into contigs (i.e., longer nucleotide sequences), and the assembly can either be performed by mapping against a known genome or *de novo* without a reference genome. Several algorithms have been developed and optimized for these assemblies to handle reads from different technologies (e.g., Velvet Optimizer or INNUca pipeline) (Zerbino and Birney, 2008; Machado et al., 2017). Assemblies are then stored in FASTA files.

During the Nanopore sequencing, the real-time base-calling (i.e., the process of converting the electric signals generated by the DNA strand passing through the nanopore protein into the corresponding base sequence) enables immediate access to results. Similar to Illumina, it provides raw data in FASTQ files subjected to quality control. Then, reads are demultiplexed to identify the reads corresponding to each specific sample. Adapters are trimmed, and sequences are aligned during the primary data analysis.

Assembly or variant calling can be performed during the second analysis phase, thanks to various bioinformatics tools (www.nanoporetech.com).

Nanopore sequencing is faster and can recognize modified bases (e.g., direct detection of DNA/RNA methylation). In contrast with Illumina technology, the Nanopore sequencing can be performed anywhere with portable devices, such as MinION™. Indeed, thanks to an automated sample preparation device called VolTRAX, the sample can be loaded directly on it. Then, a protocol is selected, and the device takes 45 minutes to prepare the sample (www.nanoporetech.com).

8.2.2.3. Whole-genome comparative analysis

Comparisons at the genome level are the most relevant strategy to define the genetic relationship between isolates at unprecedented resolution. Phylogenetic approaches based on WGS data rely on calculating genetic distances based on either single nucleotide polymorphism (SNP) or allele differences (ADs) obtained through core or whole-genome MLST (cg/wg MLST) (ECDC, 2016). SNPs analysis is performed on the whole genome, by comparison to a reference genome, or only on a set of loci (e.g., cgSNPs (Bloomfield et al., 2017)). SNPs are DNA sequence variations (i.e., a single base pair mutation at a specific locus) that occur in coding and non-coding sequences. In contrast, the gene-by-gene analysis defines allelic profiles from a set of common loci, known as the core genome, to a representative panel of isolates.

In theory, the two types of analyses could be combined to detect common source outbreaks caused by closely related or identical strains. A first clustering could be performed using a gene-by-gene based nomenclature, enabling results comparison across laboratories. It could then be followed by SNP analysis to resolve further the phylogenetic structure of identified clusters of isolates with common or closely related cgMLST profiles (ECDC, 2016). However, unlike other foodborne pathogens (e.g., *Listeria monocytogenes* or *Salmonella enterica* serovar Typhi), a high genetic diversity likely driven by HGT through homologous recombination, and to a lesser extent by chromosomal mutations, is present in *Campylobacter* populations (Boer et al., 2002; Ragon et al., 2008; Lan et al., 2009; Sheppard et al., 2011b). As a result, SNP analyses that compare strains at the nucleotide level tend to overestimate genetic exchange events and decimate the *Campylobacter* population structure signal (Sheppard et al., 2012). After conducting comparative studies between the SNP and the cgMLST approaches for different pathogens, it appears that the gene-by-gene method is more suitable for identifying lineages with this

recombining species (Dangel et al., 2019; Jajou et al., 2019). Furthermore, Including accessory loci, present in only a subsection of genomes and often associated with specific phenotypic traits of interest, improves the discriminatory power of the gene-by-gene analysis (Sheppard et al., 2012).

9. Background of the thesis project

According to the World Health Organization, *Campylobacter* spp. is one of four key global causes of bacterial diarrheal diseases worldwide (WHO, 2020a). After an increased trend of confirmed clinical cases in Europe between 2008 and 2015, the number of human campylobacteriosis has remained stable since 2015 (EFSA and ECDC, 2016, 2021a). However, the notification rate in Luxembourg, and more broadly in Europe, is still high. Some outbreaks, including 18 with strong evidence, were reported to EFSA in 2019 *via* zoonosis monitoring (EFSA and ECDC, 2021a). However, only 0.6% of human campylobacteriosis cases would be reported through food-borne outbreaks investigations (EFSA and ECDC, 2021a).

Since 2005, an integrated surveillance has been implemented in Luxembourg by collecting on a regular basis, *Campylobacter jejuni* isolates from diverse sources and by characterizing them by the MLST method (Ragimbeau et al., 2008). Then, an extended MLST method was developed by including two additional molecular markers, *gyrA* and *porA* loci (Dingle et al., 2008; Ragimbeau et al., 2014). This new typing system was validated in the framework of the HypoCamp research project (FNR, CORE C09/BM/09, 2010-2013) and was subsequently implemented in routine. Through the application of this extended MLST, the temporal distribution of the MLST+ profiles (ST-*gyr*A-*por*A) collected from human clinical isolates (N = 1,158 samples) and isolated between 2011 and 2012 was investigated. It has been revealed that 20 MLST+ profiles (3% in the whole collection of profiles) appeared regularly over years and almost 30% of the patients were infected by them. This phenomenon is intriguing as *C. jejuni* infections are mainly considered as sporadic. Additionally, sequencing the *gyrA* locus also provided information on fluoroquinolone (FQ) resistance: *gyrA* alleles 2, 3, 8, 15, 17, 30, and 44 having the C257T point mutation conferring the resistance, for instance (Ragimbeau et al., 2014). By using this sequence-based tool, collected data of over 3,000 partially characterized isolates of *C. jejuni*, isolated between 2005 and 2018 from human clinical cases, were screened to predict their resistance to FQ. Out of 108 recurring MLST+ profiles (N = 2,010 isolates) (i.e., identified at least 5 times in the database between 2005 and 2019), 59 belong to *gyrA* alleles encoding the point mutation conferring FQ resistance (1,218 isolates, n = 61% of the isolates). This was concordant with the published epidemiological data as about half of *C. jejuni* isolates were resistant to FQ in Europe (EFSA and ECDC, 2021b).

The recurrence in the *C. jejuni* genetic profiles in their temporal distribution, has suggested potential abilities to persist over space and time. However, *Campylobacter jejuni* is a strict microaerobic and well-known as a fastidious organism. Therefore, its ability to persist in environment and all along the

food processing, has long puzzled scientists. Although considered highly susceptible to oxygen concentrations, recent studies reported that some strains developed different tolerance levels regarding survival in atmospheric air (Kaakoush et al., 2007; Rodrigues et al., 2015, 2016). Indeed, they managed to cope with oxidative stresses induced by ROS formation and showed aerotolerance capability of *C. jejuni* strains, allowing them to survive in atmospheric air (Oh et al., 2015a). Few strains were even described as acclimated to aerobic conditions (i.e., they multiply in air ambient conditions) (Rodrigues et al., 2015; O'Kane and Connerton, 2017). Furthermore, it was demonstrated that strains could develop biofilms, and the formation of this structure is enhanced in aerobic conditions (Reuter et al., 2010; Ica et al., 2012; Turonova et al., 2015). These diverse phenotypical behaviors are highly variable among the pathogenic species *C. jejuni*. The wide range of responses raises the question of biological fitness among strains.

10. Objectives and scientific questions

Based on the scientific background presented, this study addresses the importance of surveillance of campylobacteriosis, assumed to be mainly sporadic in nature. Based on a long-term monitoring of partially sequenced clinical human strains, results have suggested the occurrence of diffuse outbreaks, spread over several years. This phenomenon was investigated by studying the genetic population structure of *C. jejuni* causing human infections. Hypotheses have been put forward to test what was observed under two key pillars:

- What is the temporal distribution of human clinical *Campylobacter jejuni* when genomics is applied?
- Do specific phenotypical traits contribute to the persistence pattern that was observed in the temporal distribution of these genetic profiles?

The thesis project aims to utilize state-of-the-art methodologies to answer the scientific questions. The strategies are summarized as follows:

- Comparative genomics tools will be used to assess, through the concordance of different typing schemes, the bacterial genetic material at the core genome (cg) and at whole genome (wg) levels to elucidate the population structure of *C. jejuni* in Luxembourg (chapter II).
- Phenotypical tests on a selected panel of strains performed under controlled conditions. The aim is to collect reliable data on some biological abilities previously shown to contribute to transmission and persistence in the environment (chapter III).
- Functional genomics analyses from wgMLST data to study whether particular phenotypical traits could be linked to the recurrence of certain genotypes (chapter IV).

The first chapter of this manuscript was dedicated to a state-of-the-art study to outline the socio-economic and scientific context of this research work. In the second chapter, the objectives are (i) to evaluate the concordance between different typing schemes and (ii) to compare different genotypes at the core genome and whole genome levels in order (iii) to elucidate the population structure of *C. jejuni* strains involved in human infections in Luxembourg over 13 years using WGS data. The objective of the work presented in the third chapter is to characterize the phenotype of each *C. jejuni* strain belonging to the collection. The aim is to verify if biological traits linked to potential transmission and persistence in the environment could exist (e.g., ability to tolerate oxidative stresses, multiplication in aerobic conditions, capacity to adhere to abiotic surfaces, and to develop biofilms). The fourth chapter aims to assess the potential link between the recurrence of specific genomic lineages and their phenotypical traits.

Furthermore, functional genomics analyses were performed to (i) identify specific genes present in targets shared only by one or two genomic lineages, (ii) to investigate potential correlations with mechanisms underlying phenotypical behaviors, and (iii) to potentially reveal factors that may contribute to the spatiotemporal survival of the recurrent strains. The last chapter includes a reminder of the objectives and the contextualization of the project, followed by a discussion of this thesis work results regarding the technical and scientific questions. Finally, challenges and scientific outlooks, such as the implementation of a continuous surveillance of *C. jejuni* at the European level, are discussed.

Chapter II: The genomic population structure of clinical *Campylobacter jejuni* in humans in Luxembourg

The increasing availability of large genome datasets enhances understanding of *Campylobacter* evolution through the application of several methods, such as phylogenetic analyses. A powerful evolutionary force in *Campylobacter* is genetic recombination, usually leading to the emergence of new lineages. However, despite a high level of HGT, by homologous recombination, and mutations, it has been demonstrated that *C. jejuni* populations are highly structured into clusters of related isolates called clonal complex (CC) and defined by MLST (Sheppard et al., 2012). Indeed, most of the genetic variation within *C. coli* and *C. jejuni* genotypes is the result of reassortment of known alleles (i.e., not the generation of new ones). However, the recombination rate in *C. jejuni* has not been sufficient to abolish the signals of lineages derived from a common ancestor; the population structure of *C. jejuni* is therefore described as semi-clonal (Sheppard et al., 2008). Another aspect of this structure was identified through genotyping a large number of strains from various sources by MLST. Indeed, the phenomenon of lineage segregation by host was highlighted by Sheppard *et al*. (2011) (Sheppard et al., 2011a). Some CCs are strongly associated with either broilers (e.g., CC ST-257) or cattle (e.g., CC ST-61), named host specialist, while others are commonly found in several host species and considered as host generalist (e.g., CC ST-21) (Gripp et al., 2011; Sheppard et al., 2014). This strong host-genotype relationship in *C. jejuni* was demonstrated in alleles frequencies occurring in the different reservoirs and has been used in modelling for the source attribution (Sheppard et al., 2010; Ragimbeau et al., 2014; Mossong et al., 2016; Mughini-Gras et al., 2016).

The structuring of the *C. jejuni* population, with many CC associated with particular host species, highlights the potential role that natural selective pressure also plays in determining the species' population structure. The ecotype concept was cited for describing it, an ecotype being defined as a "newly divergent and ecologically distinct population" (i.e., a clade whose members are ecologically similar to one another) (Cohan and Koeppel, 2008). It has been established that ecotypes, belonging to distinct ecological niches, are irreversibly separated from neighboring ecotypes, as the ecological barrier limits or completely prevents genetic exchanges between them. Thus, the ecological dimensions of

bacteria speciation may be identified by comparing the ecotypes (Cohan and Koeppel, 2008). According to Sheppard *et al.* (2010), several aspects of the ecotype model are relevant to the evolution of *Campylobacter* species. However, the adaptation of this species seems to be more variable than this model suggests (Sheppard et al., 2010).

Clonality is defined through the balance between vertical and horizontal gene transfers. The index of association (I_A) was originally implemented by Brown *et al.* (1980) (Brown et al., 1980). This index is "a calculation based on the ratio of variance of the raw number of differences between individuals and the sum of those variances over each locus" (Brown et al., 1980). The index ranges from 0 to 1; a value of 0 indicates the populations are completely different or separate, due to a high rate of recombination, while a value of 1 indicates no differentiation between populations (i.e., clonal population, with linkage disequilibrium, the non-random association of alleles at different loci in a given population) (Wright, 1984). Nowadays, when driven by ecological selection, clonal expansions are called selective sweeps, in which one clone outcompetes all others, purging diversity in the population (Shapiro, 2016). As mentioned in the background part in the chapter I, some genotypes appeared regularly over years in Luxembourg. However, those analyses were performed by extended MLST; it would therefore be interesting to investigate more in depth the genomes of these lineages using core genome and whole genome analysis to determine how heterogeneous they are. The genomic population structure of *C. jejuni* in humans in Luxembourg was therefore explored and is presented in the following section.

Completely or partially as in: Nennig M, Llarena A-K, Herold M, Mossong J, Penny C, Losch S, Tresse O and Ragimbeau C (2021). Investigating Major Recurring *Campylobacter jejuni* Lineages in Luxembourg Using Four Core or Whole Genome Sequencing Typing Schemes. Front. Cell. Infect. Microbiol. 10:608020. doi: 10.3389/fcimb.2020.608020.

Abstract

Campylobacter jejuni is the leading cause of bacterial gastroenteritis, which has motivated the monitoring of genetic profiles circulating in Luxembourg since 13 years. From our integrated surveillance using a genotyping strategy based on an extended MLST scheme including *gyrA* and *porA* markers, an unexpected endemic pattern was discovered in the temporal distribution of genotypes. We aimed to test the hypothesis of stable lineages occurrence by implementing whole genome sequencing (WGS) associated with comprehensive and internationally validated schemes. This pilot study assessed four WGS-based typing schemes to classify a panel of 108 strains previously identified as recurrent or sporadic profiles using this in-house typing system.

The strain collection included four common lineages in human infection (N = 67) initially identified from recurrent combination of ST-*gyrA*-*porA* alleles also detected in non-human samples: veterinary (N = 19), food (N = 20), and environmental (N = 2) sources. An additional set of 19 strains belonging to sporadic profiles completed the tested panel. All the strains were processed by WGS by using Illumina technologies and by applying stringent criteria for filtering sequencing data; we ensure robustness in our genomic comparison. Four typing schemes were applied to classify the strains: (i) the cgMLST SeqSphere+ scheme of 637 loci, (ii) the cgMLST Oxford scheme of 1,343 loci, (iii) the cgMLST INNUENDO scheme of 678 loci, and (iv) the wgMLST INNUENDO scheme of 2,795 loci. A high concordance between the typing schemes was determined by comparing the calculated adjusted Wallace coefficients.

After quality control and analyses with these four typing schemes, 60 strains were confirmed as members of the four recurrent lineages regardless of the method used (N = 32, 12, 7, and 9, respectively). Our results indicate that, regardless of the typing scheme used, epidemic or endemic signals were detected as reflected by lineage B (ST2254-*gyrA*9-*porA*1) in 2014 or lineage A (ST19-*gyrA*8-*porA*7), respectively. These findings support the clonal expansion of stable genomes in *Campylobacter* population exhibiting a multi-host profile and accounting for the majority of clinical strains isolated over a decade. Such recurring genotypes suggest persistence in reservoirs, sources or environment, emphasizing the need to investigate their survival strategy in greater depth.

1. Introduction

Campylobacter spp. is the leading cause of bacterial foodborne diarrheal disease worldwide (WHO, 2013) and the main zoonotic agent in the European Union (EU) (EFSA and ECDC, 2019). In 2018, the reported EU-wide incidence of campylobacteriosis was 64.1 cases per 100 000 population and Luxembourg had one of the highest rates in Europe (103.8) (EFSA and ECDC, 2019). *Campylobacter* is responsible for a large health and economic burden world-wide with a cost-of-illness of $1.56 billion in the USA (Scharff, 2012; Devleesschauwer et al., 2017) and 8.28 disability adjusted life years (DALYs) per 100,000 population in Europe (Cassini et al., 2018).

More than 80% of cases of campylobacteriosis are caused by *Campylobacter jejuni* and poultry is considered the main reservoir of human infections (Mughini-Gras et al., 2012; Ragimbeau et al., 2014; Mossong et al., 2016; EFSA and ECDC, 2019). Transmission is commonly associated with cross-contamination during handling of raw meat, the consumption of undercooked meat or raw drinking milk (EFSA and ECDC, 2018). *C. jejuni* lives as a commensal bacterium in the gastrointestinal tract of wild and domestic birds and mammals, including cattle and sheep. Environmental transmission routes are less frequently reported, but risks include exposure during outdoor sports, swimming in natural waters or contact with garden soil (Stuart et al., 2010; Ellis-Iversen et al., 2012; Mughini-Gras et al., 2012; Bronowski et al., 2014; Mossong et al., 2016; Kuhn et al., 2018).

Unlike for other foodborne pathogens, molecular surveillance of *C. jejuni* has not been implemented in many European countries as the majority of human infections are thought to be sporadic with a low fatality rate (0.03% in EU in 2017) (EFSA and ECDC, 2019). Nevertheless, due to the high number of reported human cases in the EU, campylobacteriosis ranks third in cause of death behind listeriosis and salmonellosis. In addition, outbreaks caused by *Campylobacter* spp. are increasingly being identified and reported on a regular basis, often linked to consumption of untreated drinking water, raw milk or chicken liver paté (Jakopanec et al., 2008; Revez et al., 2014; Davis et al., 2016; Lahti et al., 2017; Kang et al., 2019; Hyllestad et al., 2020).

The generally high incidence recorded in Luxembourg over the last decade has motivated a national implementation of molecular monitoring of *Campylobacter* circulating in food, farm animals, and environmental waters, as part of an integrated surveillance (Ragimbeau et al., 2008, 2014; Berthe et al., 2013; Mossong et al., 2016). Monitoring the *C. jejuni* population circulating in a community can function as early warning signals for outbreaks and detect long-term changes in the bacterial population, such as

emerging new virulence traits or AMR. Further, monitoring the types of *C. jejuni* in different reservoirs and environments can shed light on the epidemiology of campylobacteriosis in that region.

Initially, genotypes from the molecular monitoring were defined according to an in-house typing system originally developed for the Sanger sequencing method. This typing method consists of the seven housekeeping genes from the Multi Locus Sequence Typing (MLST) method (Maiden et al., 1998; Dingle et al., 2001) combined with allelic profiles from two additional loci: *porA* (Clark et al., 2007) and *gyrA* (Wang et al., 1993). Including *porA* and *gyrA* refines the resolution scale of MLST and creates a reliable extended MLST typing method. The *porA* locus encodes the major outer membrane protein and is highly polymorphic, but stable during human passage and within family outbreaks, making it a suitable molecular marker for epidemiologic investigations (Cody et al., 2009). Jay-Russel *et al.* (2013) supported this finding by utilizing variations in *porA* sequences as a screening tool for discriminating genetically related strains in the situation of a large outbreak (Jay-Russell et al., 2013). Interestingly, specific point mutations within *porA* were identified as markers of hyper virulence for a *C. jejuni* clone causing abortion in ruminants and foodborne disease in humans (Sahin et al., 2012; Wu et al., 2016). A sequence-based *gyrA* method was recently developed and it provides information of isolates in two respects: (i) to distinguish the major nucleotide mutation (C257T) conferring the quinolone resistance (peptide shift Thr86Ile), and (ii) to source-track clinical isolates according to a host signature in *gyrA* alleles, potentially predictive of domestic birds as source (Jesse et al., 2006; Ragimbeau et al., 2014). The discriminative power resulting from this extended MLST method indexed on a 9-loci basis is sufficient to define different lineages and human clusters (Dingle et al., 2008; Ragimbeau et al., 2014). This has recently been superseded by whole genome sequencing (WGS).

The advent of Next Generation Sequencing (NGS) technologies has significantly increased the amount of genetic information available for the characterization of bacterial isolates. Comparisons at the genome level are more relevant for defining relationships between isolates at unprecedented resolution while simultaneously allowing the full characterization of the virulome, resistome, and metabolome of the isolate. Phylogenetic approaches based on WGS data rely on calculating genetic distances based on either SNPs (single nucleotide polymorphism) or allele differences (ADs) (known as core or whole genome MLST (cg/wgMLST)) (ECDC, 2016). Unlike other common food and waterborne bacterial pathogens (*Listeria monocytogenes* (Ragon et al., 2008) or *Salmonella enterica serovar Typhi* (Lan et al., 2009)), *Campylobacter* populations display high genetic diversity likely driven by horizontal genetic exchange (Boer et al., 2002; Sheppard et al., 2011b) and to a lesser extent by chromosomal mutations. As a result,

SNP analyses that compare strains at the nucleotide level tend to overestimate genetic exchange events and, consequently, decimate the signals of the *Campylobacter* population structure (Sheppard et al., 2012). After conducting comparative studies between the SNP and the cgMLST approaches for different pathogens, it appears that the gene-by-gene approach is more suitable for identifying lineages with this recombining species (Dangel et al., 2019; Jajou et al., 2019). This gene-by-gene method defines allelic profiles from a set of common loci, known as core genome common to a representative panel of isolates. Including accessory loci, present in only a subsection of genomes and often associated with specific phenotypic traits of interest, improves the discriminatory power of the gene-by-gene analysis (Sheppard et al., 2012). For WGS analysis of *C. jejuni* and *C. coli*, several typing schemes have been developed, including two cgMLST schemes; a commercial cgMLST schema containing 637 loci from the SeqSphere+ software (Ridom GmbH, Münster, Germany; www.cgMLST.org) and the Oxford cgMLST schema with 1,343 loci (Cody et al., 2017). Two wgMLST schemes were also defined for *C. jejuni/coli* within the SeqSphere+ software (including the cgMLST and 958 accessory loci) and by the Oxford University (1,643 loci) (Cody et al., 2013). Moreover, two typing schemes were developed specifically for *C. jejuni*: a cgMLST (678 loci) and a wgMLST (2,795 loci) from the INNUENDO platform (Llarena et al., 2018). The method-dependent definition of a WGS-based genotype underlines the need for an international nomenclature to improve communication in outbreak investigation and in surveillance.

Through vigilant surveillance and molecular subtyping with extended MLST, we discovered an unexpected endemic pattern in the temporal distribution of genotypes associated with human infection over several years. The aim of this study was to investigate if these strains were indeed clonal by applying a higher resolution typing method, namely the WGS gene-by-gene approach. We simultaneously assessed the concordance between the four different typing schemes developed for *Campylobacter* spp. and their ability to separate closely related strains.

2. Materials and methods

2.1. Strain selection

Five thousand *C. jejuni* isolates, from human and non-human sources collected in Luxembourg between 2006 and 2018, were inspected. Years 2009 and 2010 were not included as no molecular surveillance data were available. Genotypic data associated with this collection included extended MLST profiles indexed on nine loci: 7 targets of MLST (Dingle et al., 2001), the partial sequence of *gyrA* (Ragimbeau et al., 2014)

and the Sequence Variable Region of *porA* (Dingle et al., 2008; Campylobacter MOMP database). The nomenclature for displaying the results of this extended MLST was defined as follows: sequence type (ST), *gyrA* (allele number), and *porA* (allele number). For example, the combination of alleles including ST19 associated with *gyrA* allele number 8 and *porA* allele number 7 is displayed as follows: ST 19-8-7.

From these, a panel of strains with identical ST-*gyrA*-*porA* profiles over four successive years was selected, including some strains with one allele variation in either the *gyrA* or *porA* loci. Care was taken to achieve a representative strain collection from all available sources (clinical, food, animal, and environmental) and years (between 2006 and 2018). Finally, we also selected a control panel of "sporadic" isolates from patients lacking a recent travel history, i.e., only domestic cases, and whose ST-*gyrA*-*porA* profile occurred only once between 2011 and 2018. This control panel was used as outgroup.

2.2. Culture, DNA extraction, library preparation, and WGS

All isolates were stored in -80°C in FBP medium (a combination of ferrous sulfate, sodium metabisulfite, sodium pyruvate and glycerol) (Gorman and Adley, 2004). For each strain, a loopful of frozen culture was spread on chocolate PolyVitex plates (BioMerieux, Marcy-l'Etoile, France) and incubated under micro-aerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 42°C for 48 h. Then, a subculture of one colony was made again on chocolate PolyVitex agar, and incubated 16 h in the above-mentioned conditions. DNA was extracted with the DNA QIAamp Mini Kit (Qiagen, The Netherlands) according to the manufacturer's instructions. DNA was quantified with the Qubit 2.0 Fluorometer (Invitrogen, Belgium) and the Qubit® dsDNA BR Assay Kit (Life Technologies, Belgium). The DNA concentration was adjusted to be within the range of 30 to 170 µg/ml for subsequent sequencing. Libraries were prepared using the Nextera™ DNA Flex Library Prep Kit or the Nextera™ XT DNA Library Preparation Kit and sequenced on the MiSeq or the MiniSeq platforms achieving either 150- or 250-bp paired-end reads. All chemistry and instrumentations are supplied by Illumina, San Diego, CA, USA. Sequenced raw reads have been uploaded to ENA and are available under the accession project number PRJEB40465.

2.3. Genome assembly and quality control (QC) criteria

For the cgMLSTs SeqSphere+ and Oxford, the paired-end raw read data were *de novo* assembled using Velvet Optimizer v.1.1.04 implemented in Ridom SeqSphere+ v6.1 (Ridom GmbH, Münster, Germany)

(Jünemann et al., 2013). Velvet Optimizer was run with automatic determination of the coverage cut-off and minimum contig length and only assemblies with >30x coverage, 1.6Mb ± 10% bp in size and maximum number of 150 contigs were included in the downstream analysis (Zerbino and Birney, 2008; Cody et al., 2017). For the cgMLST INNUENDO and wgMLST INNUENDO, the raw data were assembled into contigs using the INNUca pipeline v. 4.2.1 with default settings (Machado et al., 2017). Only profiles with no more than 2% of missing loci in either cgMLST were included in the comparative study.

2.4. WGS-based typing schemes for genome comparison

2.4.1. cgMLST and accessory schemes in SeqSphere+

For SeqSphere+, an *ad hoc* cgMLST scheme (N = 637 loci) for *C. jejuni/C. coli* developed by the commercial firm Ridom SeqSphere+ and publicly available at www.cgMLST.org was used. Details of the material and methods used for defining this typing scheme were kindly provided by Prof. Dr. Harmsen (Supplementary data S1). The cgMLST scheme consisted of 637 genes (https://www.cgmlst.org/ncs/schema/145039/locus/). Using genomic data from previously described local outbreaks, a Complex Type (CT) threshold of thirteen was defined to give guidance for delineation of possibly related from not-related genomes (Mellmann et al., 2004). In addition, cgMLST (v1.3) was merged with a screening of the alleles of the accessory genes (N = 958). Altogether, the combined typing wgMLST scheme targets 1,595 loci and the nomenclature remain the same as in the cgMLST analyses with the definition of CTs, solely based on core genome analyses, with a cluster alert of 13.

2.4.2. cgMLST Oxford scheme

Cody *et al.* (2017) designed a cgMLST scheme composed of 1,343 loci, available as an open-access and web-accessible analyses online (Jolley et al., 2018; PubMLST - Campylobacter Sequence Typing). The system assigns a unique profile ID from each isolate sequences submitted. Clustering to identify groups can be performed by selecting a threshold empirically chosen (depending on the discrimination power needed). However for this study, the scheme was implemented in SeqSphere+ for comparing strains by using an in-house nomenclature.

2.4.3. cgMLST and wgMLST INNUENDO schemes

The cgMLST and wgMLST schemes from INNUENDO include 678 and 2,795 loci, respectively, and are publicly available at Zenodo (https://zenodo.org/record/1322564#.X5l_4IhKg2y, (Rossi et al., 2018)). The cgMLST and wgMLST profiles of the INNUca assembled genomes produced in this study were called using chewBBACA suite (v 2.0.17.1) (Silva et al., 2018). The achieved cgMLST profiles were added to the cgMLST allelic profiles of the 6,526 *C. jejuni* genomes of the INNUENDO dataset, which is also available at Zenodo (Allele_Profiles/Cjejuni_cgMLST_alleleProfiles.tsv, https://zenodo.org/record/1322564#.X5l_4IhKg2y, (Rossi et al., 2018)). Minimum Spanning Trees (MST) and goeBURST distances were calculated using the goeBURST Full MST algorithm implemented in PHYLOViZ 2.0, and used to define L1:L2:L3 profiles for the cgMLST at 4, 59, and 292 loci variance (Feil et al., 2004; Francisco et al., 2009, 2012; Nascimento et al., 2017; Llarena et al., 2018). This classification system is hierarchical: L1 is the level representing the highest resolution with a threshold of 4 and it is applied for outbreak detection and investigation, L2 is the intermediate level and is used for long-term longitudinal monitoring. L3 is defined as the level with the highest concordance with the seven-gene MLST classification (Llarena et al., 2018). The wgMLST INNUENDO defines genotypes based on the combination of alleles from the 2,795 loci; no rules were initially developed for clustering isolates with similar profiles.

2.5. Comparison of the targets included in each cgMLST schemes

To crosslink loci with different naming conventions across the four typing schemes, we compared the allele sequences in a pairwise manner. Allele sequences for cgMLST SeqSphere+ were downloaded from https://www.cgmlst.org/ncs/schema/145039/. Allele sequences for cgMLST Oxford were downloaded *via* the pubMLST RESTful API (scheme 4) (Jolley et al., 2017). Allele sequences for cgMLST INNUENDO were downloaded from Zenodo (Rossi et al., 2018). We selected the first allele sequence for each loci of the four typing schemes and performed pairwise reciprocal best hit comparison for the three schemes with the rbh function of the MMseqs2 toolkit ver. 11.e1a1c (Mirdita et al., 2019) using nucleotide search including forward and reverse strand, as well as default parameters. Hits with bitscore above 100 were selected and connected across schemes with a custom script in R 3.4.4. (R Core Team, 2018) using the igraph package 1.2.5. (Csárdi and Nepusz, 2006). Sets of matching loci within the three schemes were visualized with the UpSetR 1.4.0. package (Conway et al., 2017).

2.6. Typing system concordance

The adjusted Wallace coefficient (AWC) (Wallace, 1983; Severiano et al., 2011) was used to estimate the concordance between the different typing schemes in classifying strains (Pinto et al., 2008) by the online Comparing Partitions tool (http://www.comparingpartitions.info), using the strain panel (Supplementary data S2). The degree of equivalence is reflected by AWC. It indicates the probability that two strains with the same type by one method are also categorized into the same type by another method.

2.7. Detection of wgMLST targets shared by recurrent lineages

To determine the overlap of detected wgMLST INNUENDO targets, the allelic profiles of all strains were compared. We extracted lists of targets that appeared at least once within each of the respective lineages in the collection of strains to determine and visualize overlapping and unique sets with a webtool (http://www.molbiotools.com/listcompare.html).

2.8. Cluster analyses

In SeqSphere+, *Campylobacter* isolates are classified in CTs in which the first CT assigned chronologically is definitively fixed in the database and referred to as the CT founder (Ridom SeqSphere+, 2013). In contrast, the goeBURST algorithm produces a hierarchical classification with the gene-by-gene approach and aims to predict the founder of a clonal complex based on the allele frequency in the dataset. It assumes that the ancestral genotype is the predominant one, which subsequently generates variants. To deduct and visualize the possible evolutionary relationships between strains, the goeBURST algorithm and its expansion to generate a complete MST implemented in PHYLOViZ 2.0 was used for the cgMLSTs SeqSphere+, INNUENDO, Oxford, the cgMLST SeqSphere+ combined with the accessory targets and the wgMLST INNUENDO (https://online2.phyloviz.net/index) (Feil et al., 2004; Nascimento et al., 2017).

The dynamic shared-genome based approach was performed on the MST generated for the cgMLST Oxford and the wgMLST INNUENDO in order to determine a clustering threshold. Genomic clusters were determined according to the definition of goeBURST groups, based on allelic differences ranging from 0.5 to 1% (Llarena et al., 2018). The in-house nomenclature for displaying the results of cgMLST Oxford and wgMLST INNUENDO were defined as follows: Ox+number and wg+number. For example, the Ox profile number 10 and the wg profile number 8 are displayed as follows: Ox10 and wg8, respectively. The wgMLST

profiles were used in the dynamic shared-genome based approach for the comparison and only to increase resolution for clustering strains.

3. Results

3.1. Recurrent extended MLST profiles in campylobacteriosis

By focusing solely on human clinical isolates from our historical collection (N = 3,000), we identified approximately one hundred distinct ST-*gyrA*-*porA* combinations. Two-thirds (N = 2,010) of the human strains in the collection belong to 108 main combinations (Supplementary data S3). Four lineages (ST19-8-7, ST2254-9-1, ST464-8-1678, and ST6175-9-1625, hereafter referred to as lineage A, B, C, and D, respectively) were selected due to the high number of strains (N ≥ 45) and their frequency in human infection over time (Figure 16 and Supplementary data S3). Some minor variations were accepted in *gyrA* and *porA* alleles: three variants of *porA* and one of *gyrA* in lineage A, and one variant of *porA* in lineage B (Table 3). In lineage A, the variation of *gyrA* alleles (*gyrA*1 instead of *gyrA*8) leads to the loss of the quinolone resistance (Wang et al., 1993; Payot et al., 2006). Concerning the *porA* variations, two are linked to deletions in lineage A and two to a non-synonymous mutation (one in lineage A and one in lineage B, respectively). Lineage A has appeared regularly after 2005, with an average of five strains per year and up to 23 in 2012, while 68% of all the strains belonging to lineage B were gathered in a peak in 2014 (Figure 16). For lineage C, strains displayed the same combination of alleles and occurred once in August 2008 and then reemerged from July 2014 to January 2018 (Figure 16). For lineage D, strains were characterized by the same allele combination (Table 3) and occurred once in June 2012, once in March 2014 and then regularly, from May 2016 to October 2018 (Figure 16).

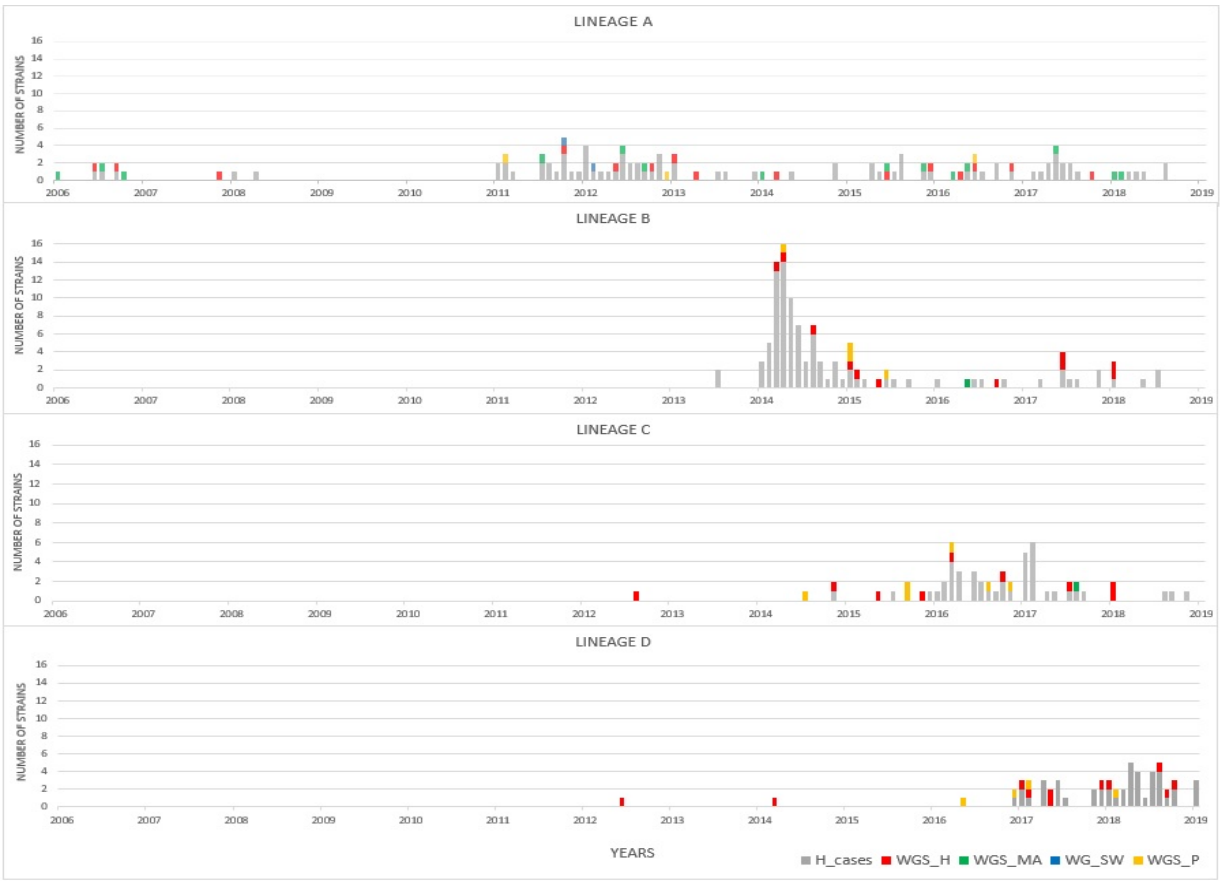

Figure 16: Distribution of strains occurrence for lineages A to D over time (extracted from (Nennig et al., 2021)).

Clinical strains of the laboratory collection are displayed in gray (extended MLST typing). Colors represent to source of selected isolates that were analyzed by WGS: human (red), cattle and sheep (green), poultry (yellow), and surface water (blue) samples (extracted from (Nennig et al., 2021)).

Table 3: Distribution of main lineages, extended MLST, and variant of the strain collection (extracted from (Nennig et al., 2021)).

Lineage	Main combination	Variants	Human	Poultry	Ruminants	Environmental	Total
A	ST 19 – *gyrA* 8 – *porA* 7		13	3	14	2	37
		ST 19 – *gyrA* 8 – *porA* 582			1		
		ST 19 – *gyrA* 8 – *porA* 2070	1				
		ST 19 – *gyrA* 8 – *porA* 2068	1				
		ST 19 – *gyrA* 1 – *porA* 7	1	1			
B	ST 2254 – *gyrA* 9 – *porA* 1		10	4	1	0	16
		ST 2254 – *gyrA* 9 – *porA* 275	1				
C	ST 464 – *gyrA* 8 – *porA* 1678		10	8	1	0	19
D	ST 6175 – *gyrA* 9 – *porA* 1625		12	4	1	0	17
Total			49	20	18	2	89

3.2. Selection of a strain panel

Overall, the selected panel included strains from various sources as the four lineages occurring in human infections were also detected in non-human samples. Altogether, the collection included isolates from human (N = 67), poultry (N = 21) and ruminant (N = 18). To complete the panel, two strains from environmental sources (surface waters) assigned to lineage A were added (Table 3). A total of 108 strains was selected for the strain panel and subjected to WGS. To achieve equal distribution of strains over the study period, strains belonging to lineage A (N = 37 of 70), lineage B (N = 16 of 97), lineage C (N = 19 of 45) and lineage D (N = 17 of 58) were selected. In addition, 19 strains with a unique ST-*gyrA*-*porA* combination were included in the panel as an outgroup (Supplementary data S2).

The acquired assemblies varied between 35x and 120x in depth of coverage and 1 to 150 contigs, associated with a percentage of good targets ranging from 98.6% to 99.8% (mean value = 99.3%) for cgMLST SeqSphere+ and from 98.0% to 99.3% (mean value = 98.4%) for the cgMLST Oxford. According to the quality criteria defined above (see Methods 2.3) as well as those of the INNUca pipeline, 15 genomes were discarded (14 with SeqSphere+ and 1 with INNUENDO criteria; 4, 1, 4 and 7 genomes were removed from lineages A, B, C and D, respectively). Consequently, genomes of 93 strains were included in the downstream analysis (Supplementary data S2).

3.3. Comparison of the loci included in the different schemes

As the number of loci selected for the core genome varies between the schemes, we compared the respective sequences to assess the number of shared loci. We compared allele sequences by reciprocal best hits. All schemes shared 432 loci, constituting the majority of targets in cgMLST SeqSphere+ and cgMLST INNUENDO with 68% and 64% of targets respectively (Figure 17). The majority of targets that differed between cgMLST SeqSphere+ and cgMLST INNUENDO was present in cgMLST Oxford. The wgMLST INNUENDO had an additional 1,775 loci not present in any of the other three cgMLST schemas (Supplementary data S4). The mean size of targets included in each cgMLST typing scheme ranges from 93 to 4,553 bp and the complete lists of targets are provided in Supplementary data S4.

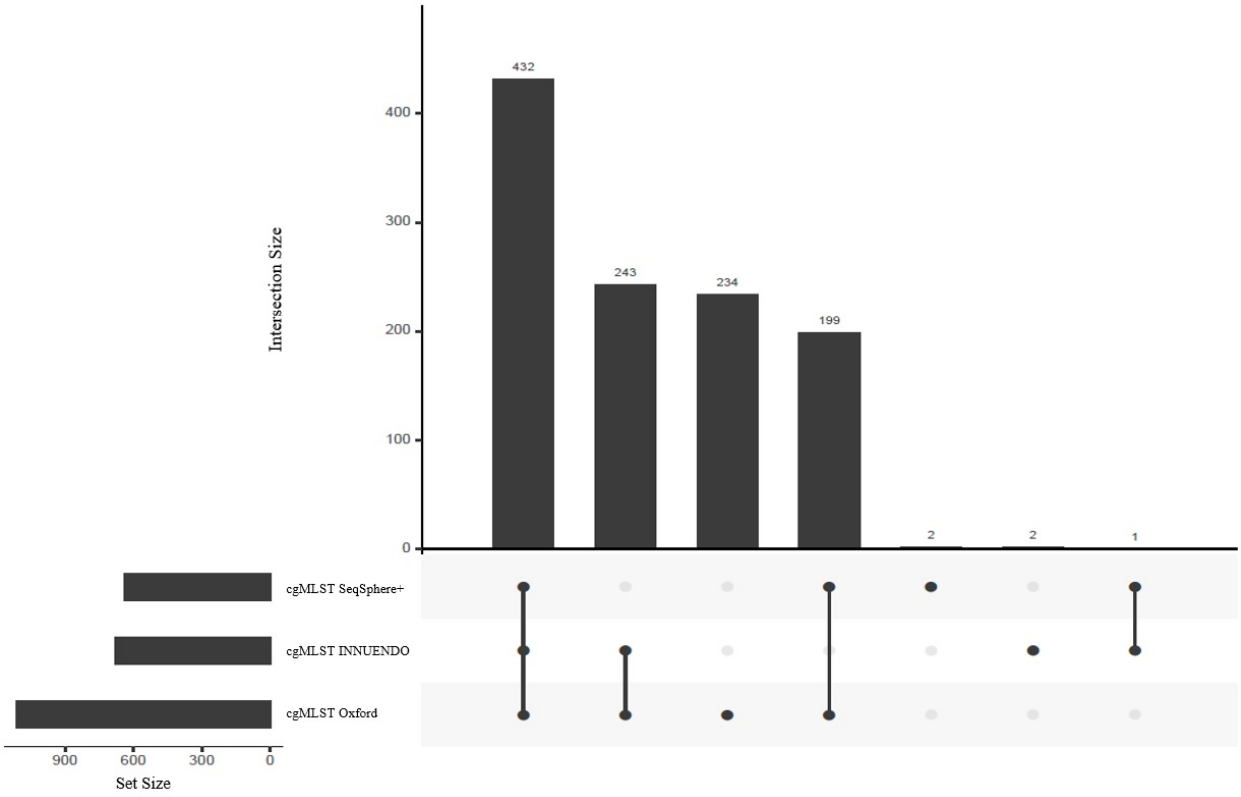

Figure 17: Shared targets between the three compared schemes: cgMLST SeqSphere+ (637 targets), cgMLST INNUENDO (678 targets), and cgMLST Oxford (1,343 targets) highlighted as set sizes (extracted from (Nennig et al., 2021)).

The central bars represent the number of shared or unique targets in or between the different schemes. The points below define the members of the respective sets. For example, 432 targets are present in all three cgMLSTs (SeqSphere+, Oxford, and INNUENDO) and 243 targets are present in both the cgMLSTs Oxford and INNUENDO but not in the cgMLST SeqSphere+. For an overview of shared targets, also refer to Supplementary Data S4 (extracted from (Nennig et al., 2021)).

3.4. Gene-by-gene WGS analysis

With the dynamic shared-genome based approach using 1% allelic differences, thresholds of 11 and 9 AD were defined to classify the strains by the cgMLST Oxford and the wgMLST INNUENDO scheme respectively (Table 4). The number of partitions, or clusters, obtained with the different methods was very close: 28 for extended MLST, 22 for cgMLST SeqSphere+, 26 for cgMLST Oxford, and 24 for cgMLST INNUENDO. The largest number of partitions (N = 32) was obtained with the wgMLST INNUENDO analysis (Supplementary data S2). From this pan-genome analysis including 2,795 targets, an average of 974 loci were detected in each lineage, with 870 loci shared between the four lineages (Figure 18).

Table 4: Characteristics of the different typing schemes to analyze WGS data from *C. jejuni* (extracted from (Nennig et al., 2021)).

Typing scheme	Number of targets	Cluster Alert distance*
Extended MLST	9	1
cgMLST SeqSphere+	637	13
cgMLST Oxford	1,343	11
cgMLST INNUENDO	678	L1: 4, L2: 59 and L3: 292
wgMLST INNUENDO	2,795	9

*The cluster alert distance is defined by a threshold value corresponding to the maximum number of different alleles between strains belonging to the same cluster.

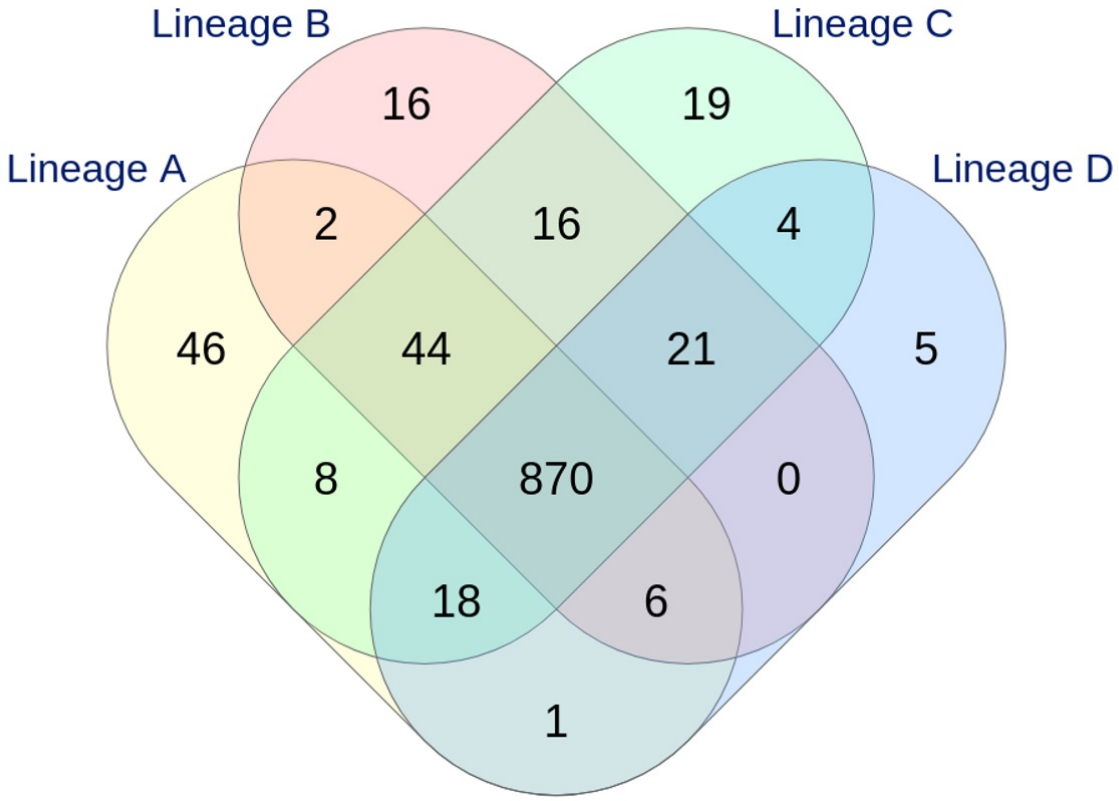

Figure 18: Venn diagram showing the relationship between the loci identified in the four lineages by wgMLST INNUENDO analysis (2,795 targets) (extracted from (Nennig et al., 2021)).

A total of 995, 975, 1,000, and 925 targets were detected in lineages A, B, C, and D, respectively (extracted from (Nennig et al., 2021)).

For the analysis of unique combinations, all sporadic strains were classified distinctly by the typing schemes, with one exception regarding two strains that were classified in the same CT (CT 1639) with cgMLST SeqSphere+, in the same profile L1:L2:L3 (66:81:1) with cgMLST INNUENDO and in the same profile with wgMLST INNUENDO (wg30). The allelic profiles for the strains generated by all typing methods were clustered and visualized in PHYLOViZ online tool, in which all five typing schemes achieved very similar unrooted MSTs (Figure 19) (PHYLOViZ Online).

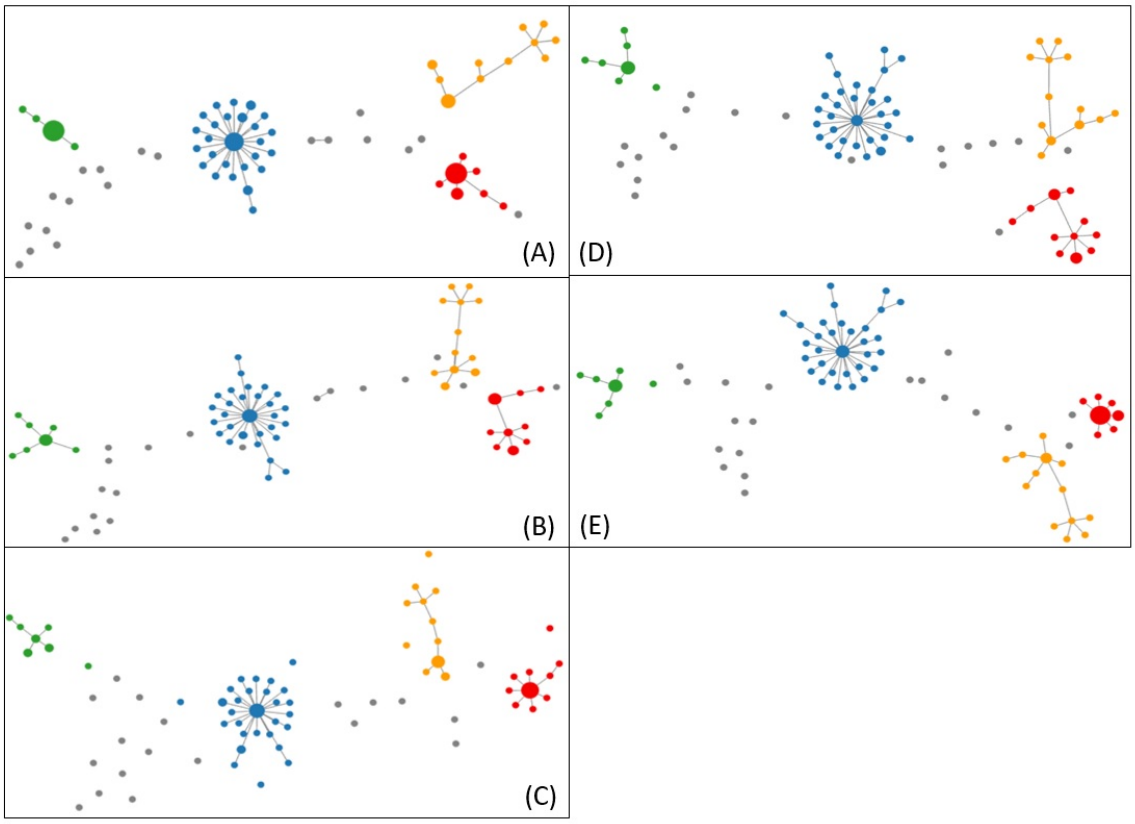

Figure 19: Minimum Spanning Trees generated using PHYLOViZ for (A) cgMLST SeqSphere+ (cut-off: 13), (B) cgMLST and accessory targets SeqSphere+ (cutoff: 13), (C) cgMLST INNUENDO (cut-off: 4), (D) cgMLST Oxford (cut-off defined by dynamic core analysis: 11) and (E) wgMLST INNUENDO (cut-off defined by dynamic core analysis: 9) analyses on tool (extracted from (Nennig et al., 2021)).

Lineage A is displayed in blue, lineage B in red, lineage C in orange, and lineage D in green and unique combinations in gray (extracted from (Nennig et al., 2021)).

Lineage A (ST19-8-7, N = 34) had very limited genetic diversity according to our gene-by-gene WGS analyses. The cgMLST SeqSphere+ assigned all strains to the same CT (CT 82), as did the cgMLST Oxford

scheme: Ox1 (Table 5). On the contrary, the cgMLST INNUENDO divided lineage A in two groups, of which the majority (33/34) were of the same L1:L2:L3 profile (1:9:1). The 34th strain had a different genotype at L1 level (2695:9:1). Thirty-two of 34 lineage A strains had an identical wgMLST INNUENDO profile: wg1, while two strains had a deviating wgMLST profile (wg5 and wg6). The strains belonging to profile wg1 were collected over a wide timespan (2006-2018) and a range of sources (human, veterinary, or environmental sources).

Table 5: Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage A (extracted from (Nennig et al., 2021)).

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp001	MA	2005	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp005	H	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp003	H	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp004	MA	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp006	H	2007	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp002	MA	2006	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp009	H	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp010	MA	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp008	SW	2011	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp014	H	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp012	MA	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp011	SW	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp015	MA	2012	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp016	V	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp018	H	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp017	H	2013	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp021	MA	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp019	H	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp020	MA	2014	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp023	MA	2015	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp029	MA	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp028	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp027	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp032	V	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp031	H	2016	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp035	H	2017	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp036	MA	2018	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp037	MA	2018	19 – 8 – 7	82	Ox1	1:9:1	wg1
Camp024	MA	2015	19 – 8 – 582	82	Ox1	1:9:1	wg1
Camp025	H	2015	19 – 8 – 2068	82	Ox1	1:9:1	wg1
Camp022	H	2015	19 – 8 – 2070	82	Ox1	1:9:1	wg1
Camp013	H	2012	19 – 8 – 7	82	Ox1	2695:9:1	wg1
Camp030	V	2016	19 – 8 – 7	82	Ox1	1:9:1	wg5
Camp034	H	2017	19 – 1 – 7	82	Ox11	1:9:1	wg6

In the column Isolation Source: H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry and SW to surface waters. ST: Sequence Type, CT: Complex Type, MLST: Multi Locus Sequence Typing, cg: core genome and wg: whole genome (extracted from (Nennig et al., 2021)).

Lineage B (ST2254-9-1) had low genetic variability according to the cg/wgMLST analyses: altogether, 15 of 16 strains had a similar cgMLST SeqSphere+ (CT 51), cgMLST Oxford (Ox2), and cgMLST INNUENDO (19:49:4) profiles. The increased resolution offered by the wgMLST INNUENDO divided the strains in three types: 75% of the strains were of wg2 while the remaining quarter was divided between wg7 and wg8. The strains belonging to the genotype wg2 were isolated from 2014 to 2018 and from diverse sources (Table 6).

Table 6: Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage B (extracted from (Nennig et al., 2021)).

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp038	H	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp041	V	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp040	H	2014	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp045	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp043	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp046	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp042	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp047	H	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp044	V	2015	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp048	H	2016	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp051	H	2017	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp052	H	2018	2254 – 9 – 1	51	Ox2	19:49:4	wg2
Camp049	MA	2016	2254 – 9 – 1	51	Ox2	19:49:4	wg7
Camp053	H	2018	2254 – 9 – 1	51	Ox2	19:49:4	wg7
Camp050	H	2017	2254 – 9 – 275	51	Ox2	19:49:4	wg8

In the column Isolation Source: H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry and SW to surface waters. ST: Sequence Type, CT: Complex Type, MLST: Multi Locus Sequence Typing, cg: core genome and wg: whole genome (extracted from (Nennig et al., 2021)).

Lineage C (ST464-8-1678) was more variable than A and B: all 15 strains were of the CT 75 and the 29:70:7 according to the cgMLST SeqSphere+ and cgMLST INNUENDO, respectively. Contrary to this, the cgMLST Oxford split the panel into three: Ox3, Ox5, and Ox6 (Table 7). The wgMLST INNUENDO discriminated six different genotypes collected from diverse range of sources between 2014 and 2017 (Table 7).

Table 7: Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage C (extracted from (Nennig et al., 2021)).

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp059	V	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp058	H	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp067	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp065	H	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp064	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp069	MA	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp063	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg3
Camp060	H	2015	464 – 8 – 1678	75	Ox3	29:70:7	wg9
Camp070	H	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg10
Camp055	V	2014	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp056	H	2014	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp066	V	2016	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp068	H	2017	464 – 8 – 1678	75	Ox3	29:70:7	wg11
Camp054	H	2012	464 – 8 – 1678	75	Ox5	29:70:7	wg12
Camp072	H	2018	464 – 8 – 1678	75	Ox6	29:70:7	wg13

In the column Isolation Source: H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry and SW to surface waters. ST: Sequence Type, CT: Complex Type, MLST: Multi Locus Sequence Typing, cg: core genome and wg: whole genome (extracted from (Nennig et al., 2021)).

For lineage D (ST6175-9-1625), all the 10 strains were gathered by the cgMLST SeqSphere+ in the same CT (CT 543), while with the cgMLSTs Oxford and INNUENDO and the wgMLST INNUENDO, one strain had a different profile from the others. The strains were isolated between 2017 and 2018 and from diverse sources (Table 8).

Table 8: Assignment of genetic profiles according to the different typing schemes for strains initially belonging to the lineage D (extracted from (Nennig et al., 2021)).

Strain ID	Isolation Source	Year of isolation	ST – *gyrA* – *porA* – Extended MLST	CT – cgMLST SeqSphere+	Genotype – cgMLST Oxford	L1:L2:L3 – cgMLST INNUENDO	Genotype – wgMLST INNUENDO
Camp082	V	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp083	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp081	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp084	MA	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp080	H	2017	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp085	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp086	V	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp087	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp089	H	2018	6175 – 9 – 1625	543	Ox4	41:68:27	wg4
Camp088	H	2018	6175 – 9 – 1625	543	Ox7	2724:68:27	wg14

In the column Isolation Source: H refers to clinical samples, MA to mammals (cattle and sheep), V to poultry and SW to surface waters. ST: Sequence Type, CT: Complex Type, MLST: Multi Locus Sequence Typing, cg: core genome and wg: whole genome (extracted from (Nennig et al., 2021)).

3.5. Concordance between the typing methods

This analysis was performed on the 93 strains selected in the panel (Methods 3.2 and Supplementary data S2). The cgMLST INNUENDO, the cgMLST Oxford, and the wgMLST INNUENDO had an AWC of 1.000 to the cgMLST SeqSphere+ schema, meaning that all strains clustering together using one of these three typing schemes are also classified together with the cgMLST SeqSphere+. The cgMLST Oxford had an AWC of 0.948 with the cgMLST INNUENDO and, conversely, the cgMLST INNUENDO had an AWC of 0.956 with cgMLST Oxford; 95% of the strains are clustered similarly using either cgMLSTs Oxford and INNUENDO. The majority (93.7% and 94.5%) of the strains that clustered with the cgMLST SeqSphere+ schema were also grouped by the cgMLST INNUENDO and the cgMLST Oxford, respectively. The wgMLST INNUENDO bundled 94.0% of the strains in a similar manner as the cgMLST INNUENDO and 99.8% as the cgMLST Oxford (Table 9).

Table 9: Adjusted Wallace coefficients values (CI 95%) for typing schemes comparison (extracted from (Nennig et al., 2021)).

	Extended MLST	cgMLST SeqSphere+	cgMLST INNUENDO	cgMLST Oxford	wgMLST INNUENDO
Extended MLST		1.000 (1.000-1.000)	0.931 (0.832-1.000)	0.935 (0.876-0.994)	0.757 (0.647-0.867)
cgMLST SeqSphere+	0.795 (0.637-0.953)		0.937 (0.843-1.000)	0.945 (0.895-0.994)	0.728 (0.596-0.859)
cgMLST INNUENDO	0.790 (0.630-0.951)	1.000 (1.000-1.000)		0.956 (0.909-1.000)	0.729 (0.597-0.862)
cgMLST Oxford	0.787 (0.622-0.952)	1.000 (1.000-1.000)	0.948 (0.852-1.000)		0.769 (0.634-0.903)
wgMLST INNUENDO	0.829 (0.658-0.997)	1.000 (1.000-1.000)	0.940 (0.828-1.000)	0.998 (0.996-1.000)	

4. Discussion

From our long-term surveillance of campylobacteriosis at national scale, our data suggested the presence of recurring genotypes defined by an extended MLST method indexing 9-loci over a 13-year period. This study investigated the relationship of a collection of isolates classified in four commonly identified lineages in Luxembourg at genome level. The aim was to assess the potential occurrence of stable genomes through the concordance of different WGS-based typing schemes exploring and comparing isolates at the core genome level (cgMLSTs from SeqSphere+, Oxford, and INNUENDO) or at the pan genome scale (wgMLST INNUENDO).

Our findings suggested that the genetic population structure of *Campylobacter jejuni* is partly composed of clonal expansion of some genotypes that persist over a long period spanning up to 13 years. Contrary to the epidemic curve commonly detected in case of foodborne outbreaks, stable genetic lineages of this pathogen could emerge to observable frequency through an endemic pattern, causing human infections on a regular basis throughout the country. In order to delineate these lineages with more confidence, efforts were focused on (i) comparing data with a robust design, and (ii) defining cut-offs values aligned with previously published data from genetic variability of the species as well as pre-established threshold for the different typing schemes tested.

Our first concern was to avoid biases generated due to low quality in the raw data and/or assemblies by establishing defined criteria before applying the gene-by-gene approach (Clark et al., 2016; Cody et al., 2017; Llarena et al., 2018; Besser et al., 2019). Quality filtering is a key prerequisite for faithful comparison of genomic data and applied criteria should be clearly stated in all WGS related reports. In their studies, Cody *et al.* (2013) and Kovanen *et al.* (2014) implemented a quality threshold in filtering the length of the reads with fixed criteria before the assembly. In 2017, Cody *et al.* applied a maximum of 150 contigs covering at least 95% of cgMLST targets (Cody et al., 2017), whereas the INNUENDO pipeline included a QC step requiring an assembled depth of coverage of 30x associated with at least 98% of scheme targets found in the cgMLST analyses (Llarena et al., 2018). The Draft Standard of International Standardization Organization (ISO/DIS 23418) suggests a depth of coverage of at least 20x for Illumina short-read raw data and 95% of the read lengths should be over 120 bp (International Organization for Standardization) (International Organization for Standardization) depending on the application. In our analyses, we implemented stringent criteria for quality filtering to ensure robustness and minimizing potential biases related to missing targets generated by poor quality sequencing data.

While a core genome of a bacterial species is expected to consist of a conserved panel of functional genes (also properly called housekeeping genes), mostly present in the genomes of interest and essential to the microorganism, the cgMLST of the three tested methods included a different number of loci. This discrepancy resulted from a more or less stringent definition of the core genome applied to a panel of reference genome varying in size and quality. It is also noteworthy that the cgMLST INNUENDO schema was specifically determined from the *C. jejuni* species while the two others have included some *C. coli* genomes to create their schemes. In summary, SeqSphere+ and INNUENDO selected targets present in at least 90% of the complete genomes (N = 12) or in 99.9% of draft genomes (N = 6,526), respectively (Llarena et al., 2018). The cgMLST Oxford was built from loci occurring in 95% of the *Campylobacter* sp. reference panel (N = 2,472) to take into consideration variation in sequence quality and applied algorithms (Cody et al., 2017). They proposed a more relaxed core genome definition as some isolates may contain mutations, leading to the reduction of the core genome size as more isolates are selected, and that analyses conducted on incomplete draft genomes might constitute a source of missing data (Cody et al., 2017). The finalized cgMLST Oxford scheme represents thus 82% of the reference genome NCTC11168, which places this typing scheme as an intermediate between a core genome and a whole genome MLST scheme with a total of 1,343 loci vs. 637/678 for the two others. A sample-set

independent approach was recently proposed to select a conserved-sequence genome as a novel core genome methodology to address this issue (Van Aggelen et al., 2019).

Further, the locus definition is different in the various schemes as well as allele calling algorithms. In the so-called gene-by-gene approach, a locus does not necessarily correspond to the complete coding sequence of a gene but can constitute a specific region. Thus, each schema includes target sequences of varying length ranging from 100 bp to several kb. Surprisingly, the sizes distributions of the targets in the three cgMLSTs tested are very similar with approximatively: 21% below 500 bp, 40% ranging from 500 to 1,000 bp, 26% ranging between 1,000 and 1,500 bp, and 13% above 1,500 bp. Except for the SeqSphere+ commercial platform, the design of allele-calling pipelines from the two others WGS-based schemes were published (Jolley et al., 2018; Silva et al., 2018). Both define alleles from sequence assemblies but perform a search by using nucleotide or translated sequences with BLASTN or BLASTP queries and by using "exemplar alleles" as reference or all alleles already recorded in the database. The procedure differs mainly when new sequences display no exact match with known alleles. However, both pipelines validate the nucleotide sequence after translation of DNA codons and include a threshold in percentage sequence identity and length.

A predefined allele distance threshold allows assignment of an unique identifier to genomes displaying a high level of similarities in their cg/wgMLST profiles. The cut-off distance value for distinguishing clusters is expressed as a number of ADs and is species or even lineage-specific. To calibrate this value, a test population commonly includes clonal outbreak strains as well as non-epidemiologically linked outgroups. Therefore, the established thresholds are based on strains collected over a relatively short period, and may thus not be appropriate for long-term surveillance. Genomic variations linked to insufficient sequencing quality and microevolutions generated during the gut passage are taken into account for classifying strains (Cody et al., 2013; Revez et al., 2013; Thomas et al., 2014; Barker et al., 2020). For instance, Cody *et al.* (2013) observed between 3 to 14 loci differences (of 1,643 loci in total) in *Campylobacter* sp., during human gut passage, mainly restricted to insertions and deletions in homopolymeric tracts in contingency loci regulating phase variations of surface structures (Jerome et al., 2011; Barker et al., 2020). To classify related-genomes, Cody *et al.* (2013) tested two methods: a hierarchical approach based on an increasing number of loci in order to detect closely related isolates and a pairwise comparison based on 1,026 loci shared by the 379 *C. jejuni* genomes analyzed. Their results lead to the conclusion that the hierarchical approach is better suited to examine isolates epidemiologically related, while pairwise comparisons are preferable for the identification of outbreaks without initial

suspicion (Cody et al., 2013). We assessed genomic clusters in our WGS data with goeBURST and we found that defined low cut-off values ranging from 6 to 11 AD and from 5 to 9 AD were appropriated to classify profiles generated with cgMLST Oxford and wgMLST INNUENDO schemes, respectively. By utilizing our newly established thresholds, the classification was consistent with the ones created by cgMLST methods that use a predefined threshold like SeqSphere+ (AD = 13 of 637 targets) and cgMLST INNUENDO (AD = 4 of 678 targets).

Overall, a high concordance in clustering strains was observed between the three cgMLST typing schemes, although congruence is higher between the cgMLSTs Oxford and INNUENDO schemes (predictive of each other in 95% of the cases) compared to the SeqSphere+ scheme. This was not expected, at first glance, as cgMLST schemes from SeqSphere+ and INNUENDO have a close number of targets (637 vs. 678 targets, respectively) and share 68% of loci. The concordance between the cgMLST schemes Oxford and INNUENDO, both defined from a large collection of strains, suggests a more representative and stably defined core genome. It is noteworthy that in this study, the added value of the number of loci in the cgMLST Oxford cannot be truly attributed on its discriminative power as the datasets contain several clonal population. A largest test population, reflecting the genetic diversity within the *C. jejuni* species, would have been more appropriate for evaluating the resolution of the different typing schemes. As expected, cgMLST profiles could not be mapped with confidence to the wgMLST INNUENDO profiles including a significant larger number of targets. Differences in the accessory genome composition or in the allelic variations could explain these discrepancies. As all the lineages selected for this study originated from various hosts, it could be interesting to further investigate on a possible link between accessory genomes and niche adaption (Woodcock et al., 2017).

The clonality signal appearing through the concordance of the different typing schemes in classifying strains supports the idea of stability of these clones over time and sources. Two independent studies introduced the concept of monomorphic genotypes for *C. jejuni* within the generalist lineages Clonal Complex (CC) ST-21 (Wu et al., 2016) and ST-45 (Llarena et al., 2016). The first study investigated the genetic basis responsible for the hyper virulence of a known clone named “sheep abortion” (clone SA, ST-8), causing foodborne illnesses in human and ruminant abortion (Wu et al., 2016). The second study explored the population structure of the generalist ST-45-CC, overrepresented in human cases in Finland (Llarena et al., 2016). Considering another field, clonal expansion linked to the acquisition of antibiotic resistance has also already been highlighted in *Campylobacter* (Wimalarathna et al., 2013). Observing stable genotypes in *Campylobacter jejuni* over time are in accordance with these results, hypothesizing

that predominant clonal evolution is a major adaptive evolutionary strategy in microbial pathogens (Tibayrenc and Ayala, 2017).

In our study, the best example for stable genome over time is lineage A (ST19-*gyrA*8-*porA*7) as its recurrence occurs over more than a decade, although at a low level, representing an average of 13.4% of human cases per year (data not shown). Thirty-two strains of 34 from diverse sources (human, cattle and sheep, poultry, and environmental samples) were gathered in the same genetic profile at the whole genome level. This result reflects that this lineage is likely derived from one common ancestor, which thereafter disseminated broadly to a variety of mammals and birds, clearly demonstrating an ability to disperse in the environment and adapt to different ecological niches. Thus, the question of the environmental transmission routes arises, particularly concerning animal reservoirs such as poultry and ruminants that could contribute to water contamination (Mughini-Gras et al., 2016). Persistent strains have already been identified, mainly in poultry farms and in milk, and it would be interesting to link lineage A with other contamination sources such as insects, rodents, drinking water, or the surrounding environment (Kudirkienė et al., 2010; Perez-Boto et al., 2012; Rauber-Würfel et al., 2019; Jaakkonen et al., 2020).

The lineage B (ST2254-*gyrA*9-*porA*1) arose unexpectedly from our national surveillance with an epidemic curve between March and April 2014 (>70 campylobacteriosis cases). Interestingly, after this episode, clinical isolates of this lineage were still collected but at a much lower frequency during the following four years. To put things into context, this particular ST was singular in 2014 and by querying the pubmlst.org database (Jolley et al., 2018); only a dozen strains had been recorded at that time including two from poultry origin. Interestingly, the same “clone” was finally isolated in the framework of the official controls conducted by the state veterinary laboratory in Luxembourg and supported chicken as a possible source of this outbreak. In molecular epidemiology, the expression “clone” generally refers to a set of independently isolated microbial organisms that have similar genotypic traits as a results of a shared common ancestor (Van Belkum et al., 2007). The analysis using different typing schemes gathered 80% of the tested strains from lineage B in the same CT, whereas only 50% of isolates from lineage C formed a cluster. These data support the occurrence of the most large-scale outbreak caused by *C. jejuni* ever identified in Luxembourg and linked to chicken imported from neighboring countries, as the local production is negligible. Two years after the epidemic episode, this clone was isolated from a bovine source for the first time, while the remainder of lineage B was mainly isolated from poultry. The extent of

ecological niches suggests that strains from lineage B were able to cross ecological barriers and disseminate in the environment with a generalist profile (Sheppard et al., 2014).

Lineage C (ST464-*gyrA*8-*porA*1678) displayed two micro-epidemic peaks: one in March 2016 and a second in January and February 2017. Since then, its incidence has been low with less than 10 human cases per year since March 2017 and we observed a first sample of bovine source isolated in August 2017. An average of two human cases per month from December 2016 to May 2018 indicates the profile of an emerging clone tending to have an endemic profile. Notably, this lineage displays the *gyrA* allele 8, one of the nucleotide allele in *C. jejuni* containing the C257T mutation (peptide shift Thr86Ile) which confers quinolone resistance (Ragimbeau et al., 2014). Indeed, dispersion of antimicrobial resistant lineages due to positive selection was previously described for bacterial pathogens, such as uropathogenic *Escherichia coli* (ST 131 for example) (Totsika et al., 2011; Yamaji et al., 2018) and *C. jejuni* (ST 464 for instance) (Cha et al., 2016). For lineage D (ST6175-*gyrA*9-*porA*1625), the first isolate was identified in 2012 from a human infection, then in 2014 and at the beginning of 2016. A link with poultry source was observed.

Whatever the typing scheme used, clear signals appeared in our molecular surveillance for identifying an outbreak (lineage B in 2014) or the phenomenon of recurrent clones, which cause of more than 50% of human infections in Luxembourg. This study provides new insights for the genomic surveillance of *Campylobacter* infections. Through the exploration of the large collection of data that we have initiated 15 years ago, we seek to demonstrate the strong interest in monitoring genotypes causing gastroenteritis in the sense that campylobacteriosis is not only of sporadic nature. A recent study based on collected WGS data in Denmark also supported these findings (Joensen et al., 2018).

Molecular surveillance of foodborne pathogens is currently implemented for *Salmonella* (Dangel et al., 2019), *Listeria* (Van Walle et al., 2018), and VTEC (Joensen et al., 2014) at the EU level (ECDC, 2019b) and in the USA (Ribot et al., 2019). (Ribot et al., 2019). For *C. jejuni*, such monitoring in routine is hindered by the absence of a validated scheme at international level and the lack of evidence for the spread possibility of cross-border genotypes. The presence of recurring genotypes highlights the possible long-term existing of stable clones representing a risk factor of geographic spread that needs to be investigated further. Like for the acquisition of antibiotic resistance, persistent strains may have acquired specific phenotypic traits to adapt to other hosts or disperse in the environment. Habituation to ambient air (Rodrigues et al., 2015, 2016), adhesion to inert surface (Sulaeman et al., 2010; Oh et al., 2016), and biofilm formation (Reuter et al., 2010; Turonova et al., 2015) could contribute to the survival strategies of *C. jejuni* in the environment. In the future, studying the phenotypic traits of recurrent clones and their

relationship to spatiotemporal persistence would broaden our understanding on *Campylobacter* adaptation and its transmission to humans.

Data availability statement

Sequenced raw reads have been uploaded to ENA and are available under the accession project number PRJEB40465.

Supplementary material

The Supplementary Material for this article is published and can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2020.608020/full#supplementary-material.

Chapter III: Survival and persistence-related phenotypes among the genomic lineages

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1. Introduction

The genomic comparison analyses revealed that *C. jejuni* strains could be segregated in distinguished genomic lineages with a higher occurrence in human infection and a more stable genome over the years (chapter II). As the transmission from primary reservoirs to human requires adaptation capabilities to overcome harmful, even lethal, conditions, this chapter is dedicated to the investigations of features that could favor survival, adaptation, and persistence of *C. jejuni*. These behaviors are often related to phenotypes relying on the set of observable characteristics or traits of an organism. The first objective was to select the representative phenotypes of *C. jejuni* that could contribute to these resilient behaviors and be applicable and observable in lab-controlled conditions.

Considering the particularly fastidious growth *in vitro* of *C. jejuni* (37-42°C, ~5-6% O_2, and ~10% CO_2), the question arises about its ability to pull through under stressful conditions *in situ*, i.e. on food and carcasses. It has to develop survival mechanisms that could play a role in transmission through the environment, throughout the food chain, and host colonization to trigger human infection (Bronowski et al., 2014; Yahara et al., 2017). These mechanisms include protection against oxidative stresses in aerobic conditions (AC), survival to heat shock, and adaptation to low pH (Kim et al., 2021). According to Pascoe *et al.* (2015) and Karki *et al.* (2019), the primary strategies used by *Campylobacter* for its persistence are the formation of biofilms and the ability to survive oxidative stresses (Pascoe et al., 2015; Karki et al., 2018). Therefore, the bacterial biofilm is recognized as a key factor in the survival of *C. jejuni* in various ecological niches (Joshua, 2006) and some strains of *C. jejuni* have been reported to have different tolerance levels in the presence of atmospheric oxygen concentrations (Kaakoush et al., 2007; Rodrigues et al., 2015, 2016; Oh et al., 2017). As a few strains have been described in the literature as adapting and

multiplying in AC up to date, this criteria also drew our attention (Rodrigues et al., 2015; O'Kane and Connerton, 2017).

This study aimed to investigate the phenotype of 83 strains of *C. jejuni*, isolated from multi-hosts between 2005 and 2018 in Luxembourg. The objective was to determine whether these strains could (i) survive oxidative stresses, (ii) adapt to aerobic conditions, (iii) adhere to an inert surface, and (iv) develop biofilms. These specific phenotypical traits were investigated as they could be potentially linked to strain survival and persistence over time.

2. Material and methods

2.1. Bacterial strains and growth conditions, and genotyping data

The panel included strains from various sources (human, mammals, poultry, and environment). As the first objective was to select the representative phenotypes of *C. jejuni* that could contribute to resilient behaviors, 71 strains from our previous study were selected as they belong to recurrent lineages (i.e., endemic, epidemic, and emergent) (n = 52) and clinical sporadic ones (n = 19) (Chapter II). To achieve a better distribution of strains over the study period, the collection was completed with 10 sporadic strains from environmental sources. In addition, one reference strain (NCTC 11168), and the Bf strain (an atypical aerotolerant strain) (Rodrigues et al., 2015) were chosen. A total of 83 strains was selected and subjected to the defined phenotypical tests (Appendix A).

The strains were stored at -80°C in the FBP medium (a combination of ferrous sulfate, sodium metabisulfite, sodium pyruvate, and glycerol) (Gorman and Adley, 2004). Before each experiment, for each strain, a loopful of frozen culture was spread on Karmali agar (Oxoid, Deutschland) or BD™ *Campylobacter* Bloodfree Selective Medium (Becton Dickinson GmbH, Deutschland), and incubated under microaerobic conditions (MAC) (6% O_2, 3.6% CO_2, 3.6% H_2, and 86.8% N_2) at 42°C for 48 h. Then, a subculture was obtained from one colony on the same medium and incubated for 16 h in the conditions mentioned above.

WGS data associated with this strain panel were collected from our previous study (see Chapter II). For the additional strains, DNA extraction, library preparation, sequencing analyses, and quality control were performed as previously described (see Chapter II). WGS data were analyzed with the cgMLST Oxford scheme composed of 1,343 loci and available online as an open-access tool (Cody et al., 2017; Jolley et

al., 2018; PubMLST - Campylobacter Sequence Typing). This scheme was implemented in SeqSphere+ v6.1 (Ridom GmbH, Münster, Germany) to compare strains using the same previously defined in-house nomenclature, i.e. with a cut-off alert set at 11 (see Chapter II). MLST data were extracted from cgMLST data to determine the STs and CCs. Regarding the strain distribution by CCs, 34 strains belonging to CC ST-21 (lineages A and D, n = 22 and 12, respectively), 10 to CC ST-257 (lineage B), and 8 to CC ST-464 (lineage C) were selected (see Chapter II). Additionally, eight sporadic clinical strains belonged to CC ST-21, 4 to CC ST-48, and one to CC ST-257. CC STs-42, -45, -206, and -354 were represented by one sporadic human strain, while one sporadic veterinary strain belonged to CC ST-353. CC STs-179, -607, and -952 were represented by one environmental sporadic strain. Bf and NCTC 11168 belong to CC ST-403 and ST21, respectively. STs from eight sporadic environmental strains remained unassigned to CC.

2.2. Survival of *C. jejuni* isolates in oxidative stress conditions

The strains were exposed to three concentrations (0.12, 0.25, and 0.50 mM) of paraquat (PQ) or hydrogen peroxide (H_2O_2) solutions to induce superoxide and peroxide stresses as previously described by Rodrigues *et al.* (2015) (Rodrigues et al., 2015), with the following modifications: after resuspension, the cultures were standardized to an optical density at 600 nm (OD_{600nm}) of 1.00 (≈ 10^9 CFU.mL^{-1}) and diluted in sterile peptone water to obtain a bacterial suspension containing approximately 10^6 CFU.mL^{-1}. The initial culture was plated out for concentration determination: an average concentration of 9.5 ± 0.3 $\log_{10}$ CFU.mL^{-1} (n = 83) was determined. The bacterial suspensions were incubated for 45 min at 42°C in a modular atmospheric controlled system cabinet in microaerobic conditions. The capacity of the strains to resist oxidative stress was determined by plating dilutions on Karmali agar plates using the microdroplet technique as described by Rodrigues *et al.* (2015) (Rodrigues et al., 2015). The survival rate was calculated as the viable cell concentration obtained after oxidative stress divided by the initial concentration. The experiment was performed in three technical replicates and an average of the triplicates values was calculated. A control was carried out in parallel with each test, containing the same bacterial inoculum but without the addition of PQ or H_2O_2.

2.3. Acclimation to aerobic conditions

According to Rodrigues *et al.* (2015), cells were prepared for acclimation to AC by growing them in MAC on charcoal-based selective medium for 48 h (Rodrigues et al., 2015). Colonies were then harvested and resuspended in sterile peptone water and standardized to an OD_{600nm} of 1.00 (≈ 10^9 CFU.mL^{-1}). For each bacterial suspension, 1 mL containing approximately 10^9 CFU.mL^{-1} was inoculated onto charcoal-based selective agar plates and incubated at 37°C under AC for 48 h. Then, all the recovered colonies were subcultured two times successively on charcoal and blood free Columbia base agar (Biokar, France) plates in parallel and incubated at 37°C for 48 h in AC. The initial culture, initially resuspended in sterile peptone water, was plated out for concentration determination: an average concentration of 9.5 ± 0.3 $\log_{10}$ CFU.mL^{-1} (n = 83) was determined. Colonies were submitted to the MALDI-TOF Biotyper Microflex LT (Bruker Daltonics, Germany), associated to *in vitro* diagnostic and research use only databases, for identification after the third subculture in AC, and the genome of seven of them was sequenced using Illumina technology. Part of these colonies were also stored at -80°C in the standard conditions.

2.4. Adhesion to an inert surface and biofilm formation

The adhesion capacity to an inert surface of *C. jejuni* strains was determined using the BioFilm Ring Test® (KITC004, BioFilm Control, France). The protocol was adapted from the one described by Sulaeman *et al.* (2010) (Sulaeman et al., 2010). Briefly, each culture, cultivated as described in 2.1., was harvested and resuspended in ultrapure water (UPW) (Water UHPLC-MS, code product 15339865, Thermo Scientific); the OD_{600nm} was adjusted to 1.00 (≈ 10^9 CFU.mL^{-1}). The initial concentration estimated at 10^9 CFU.mL^{-1} was determined by plate counting. The toner solution (TON004) containing magnetic beads was mixed for 1 min and added (10 µL.mL^{-1}) to each calibrated bacterial suspension. A quantity of 200 µL of bacterial suspension mixed with the beads was distributed in 96-well microtiter plates, with three wells per strain (technical replicates). The plates were incubated for 2 h in AC at 42°C. After incubation, an inert opaque oil was used as a contrast liquid, and few drops covered the bacterial solution in the wells. Then, microplates were magnetized for 1 min with the magnet block and read using the dedicated Scan Plate Reader operated with a computer through BIOFILM CONTROL ELEMENTS® 3 software in a custom mode (BioFilm Control, France). The calculated numerical value, called Biofilm Formation Index (BFI), measures the aggregation density of the toner beads under the effect of a magnetic field generated by a magnet. As described by Sulaeman *et al.* (2010), the ΔBFI ($BFI_{control}$ – BFI_{sample}) was calculated to express the adhesion capacity of strains (Sulaeman et al., 2010). Three wells were filled up with ultrapure water and

beads only for each microplate, representing the negative control. For each strain, three biological replicates with three technical replicates each were performed. Values were considered valid when the standard deviation between technical replicates did not exceed 1.5.

2.5. Biofilm formation

A reliable *in vitro* protocol was specifically designed to characterize biofilm-producing *C. jejuni* strains using the BioFilm Ring Test® technology. This approach is developed in the result section. Detection of biofilm was then determined using the same tools as abovementioned for adhesion and expressed as ΔBFI.

2.6. Statistical analyses

Statistical analyses included the comparison of groups of strains classified on their survival to oxidative stresses (H_2O_2 and PQ), their acclimation to aerobic conditions, their adhesion capacity, and their biofilm formation ability. Results were analysed with JMP v.15 software (SAS Institute Inc., North Carolina, USA), and Microsoft® Excel® 2016 (v.16.0.5239.1001), using the chi-squared test for qualitative variables and variance analysis (ANOVA) for quantitative variables. The significance level was determined at 95%, $p<0.05$ considered as significant. When multiple comparisons were performed, the confidence levels for each comparison performed have to be higher, so that the result of the multiple comparisons meets the 95% confidence level. For this purpose, the Tukey-Kramer method was used to keep the alpha risk at 5%. The relative risk was also calculated after each chi-squared test. Distributions were displayed using box and whisker diagrams for graphical representation.

3. Results

3.1. Survival to oxidative stress

Two pro-oxidant reagents, PQ and H_2O_2, were used to induce superoxide and hyperoxide stresses, respectively. Overall, diverse responses were observed among strains after a short contact with different concentrations of PQ and H_2O_2 (Figure 20, Figure 21). A total of 18, 28, and 17 strains survive to 0.12 mM, 0.25mM, and 0.50 mM of PQ, respectively. No survival was observed for 20 strains in the presence of 0.12

mM of PQ (Figure 20). A total of 27, 23, and 8 strains survive to 0.12 mM, 0.25 mM, and 0.50 mM of H_2O_2, respectively. In the presence of 0.12 mM of H_2O_2, 25 strains could not survive (Figure 21). In summary, 38 and 52 strains were not able to cope with a concentration equal to or above 0.25 mM of PQ and H_2O_2, respectively (Figure 22). They have been classified as 'susceptible' (Table 10). In contrast, 45 and 31 strains could deal with higher concentrations (0.25 or 0.50 mM) of PQ and H_2O_2, respectively (Figure 22). They were consequently assumed to be the best survivors and were classified as 'resistant' (Table 10).

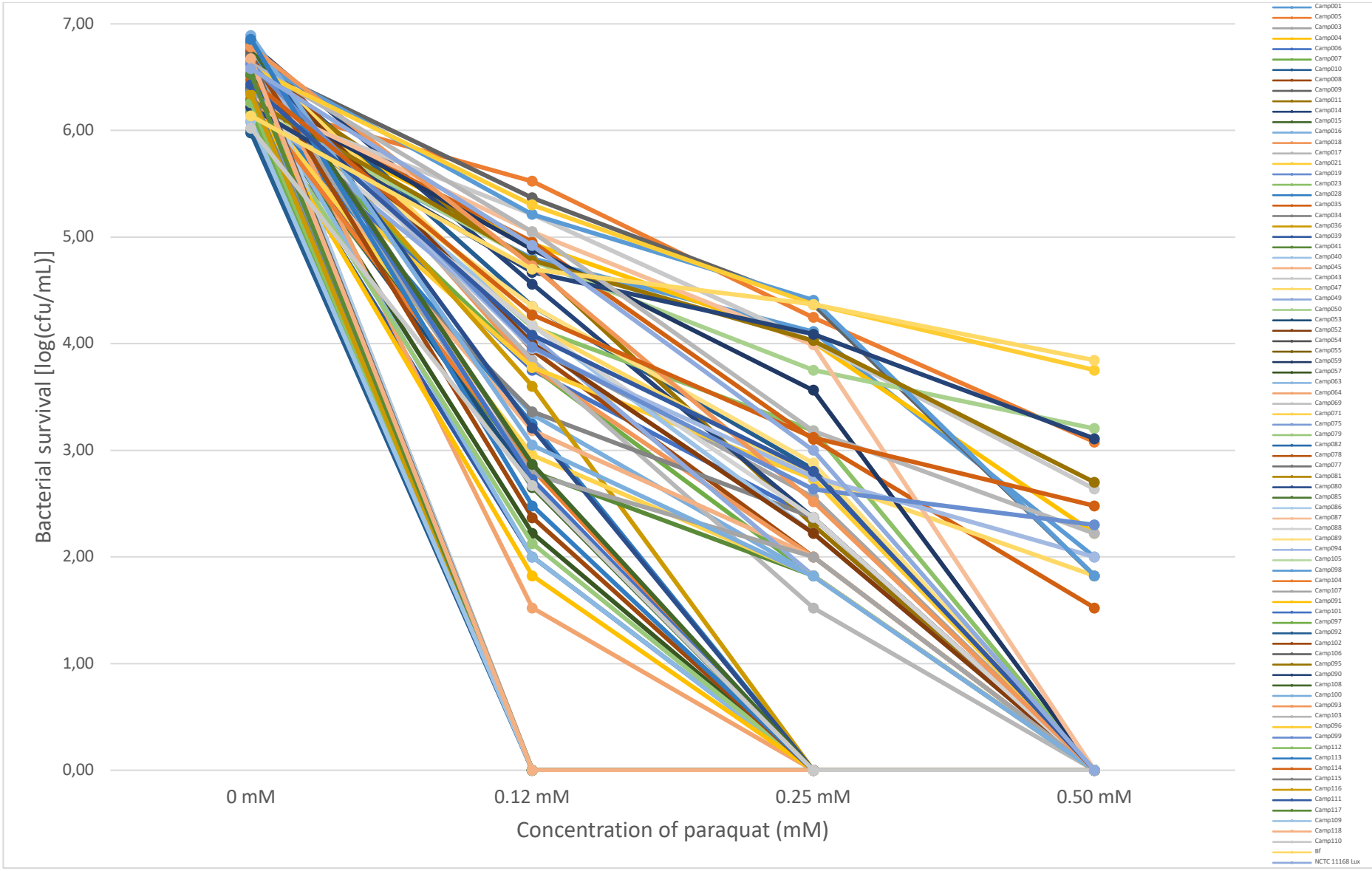


Figure 20: Response of 83 *C. jejuni* strains to 0, 0.12, 0.25, and 0.50 mM of superoxide stress using paraquat (PQ).

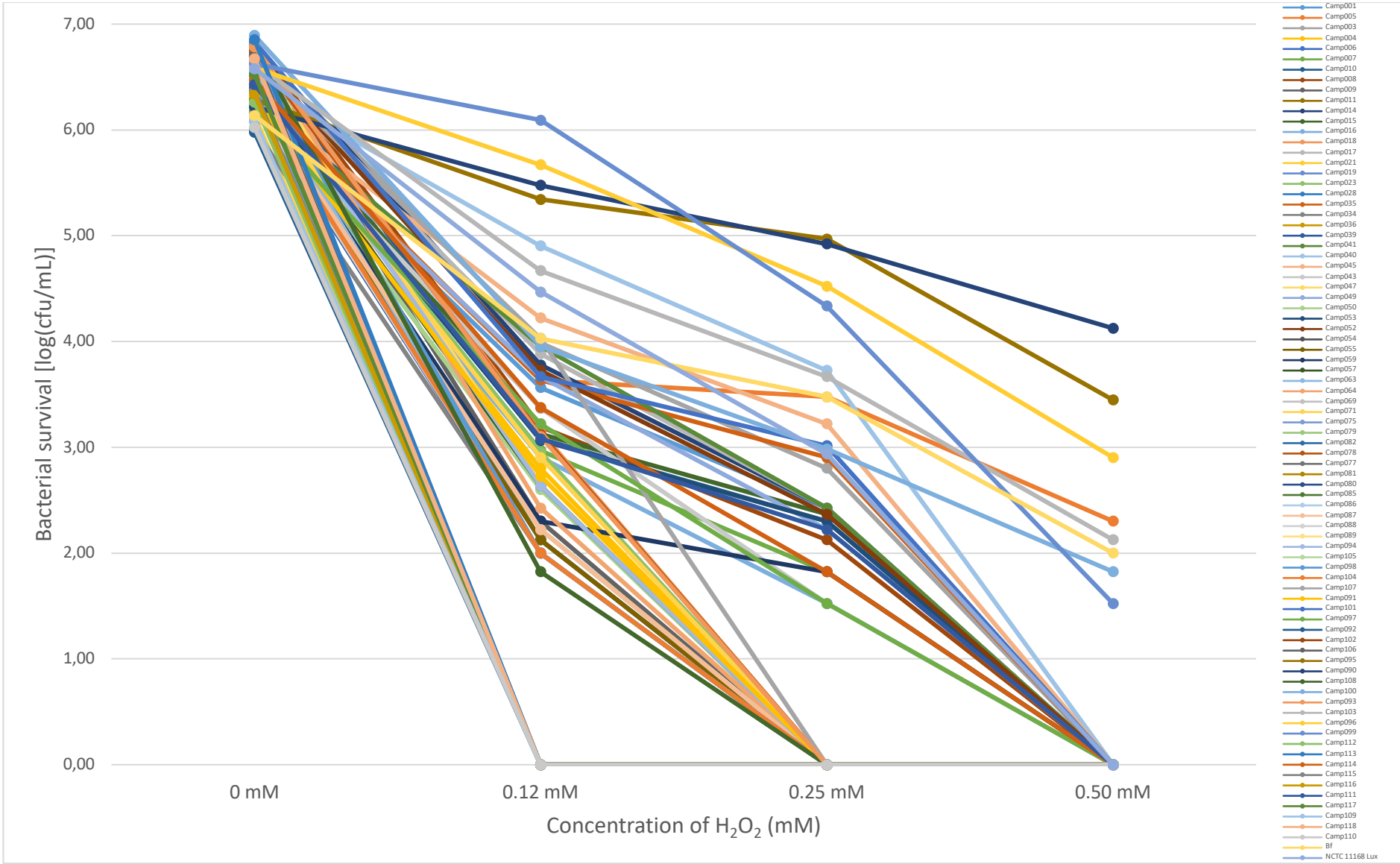


Figure 21: Response of 83 *C. jejuni* strains to 0, 0.12, 0.25, and 0.50 mM of hyperoxide stress using hydrogen peroxide (H_2O_2).

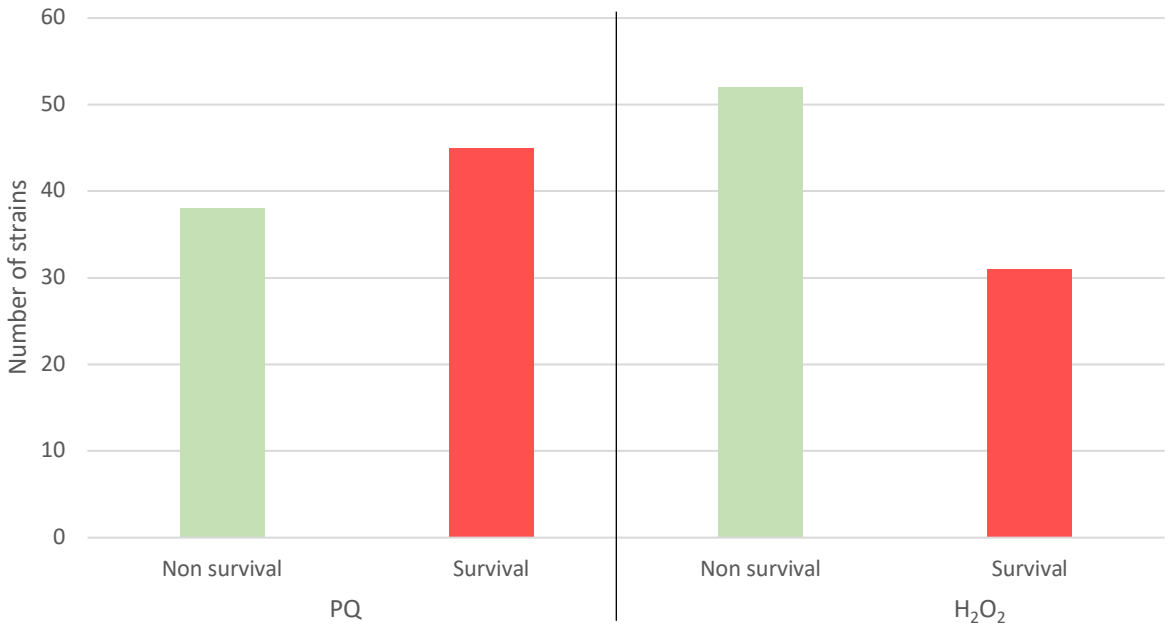


Figure 22: Survival of strains at or above 0.25 mM of superoxide (PQ) and hyperoxide (H_2O_2) stresses.

Table 10: Classification of 83 *C. jejuni* isolates according to their response to superoxide and hyperoxide stresses.

Concentrations tested	Number of strains surviving the superoxide stress (PQ)	Number of strains surviving the hyperoxide stress (H_2O_2)	Classification
< 0.12 mM	20	25	Susceptible
0.12 mM	18	27	
0.25 mM	28	23	Resistant
0.50 mM	17	8	

3.2. Correlation between responses to superoxide and hyperoxide stresses

Statistical analyses to calculate this correlation were based on qualitative variables for the chi-squared test (i.e., the survival or not of strains exposed at ≥ 0.25 mM PQ or H_2O_2). They were performed on quantitative variables for the ANOVA test (i.e., the survival rate of strains exposed at ≥ 0.25 mM PQ or H_2O_2). Interestingly, at equivalent molarity (0.25 mM) PQ is not significantly more harmful than 0.25mM H_2O_2. The survival at 0.25 mM PQ was significantly associated with the survival at 0.25 mM H_2O_2 (ANOVA, $p < 0.0001$) (Figure 23), 27 strains exhibiting survival to both stresses at 0.25 mM (Figure 24).

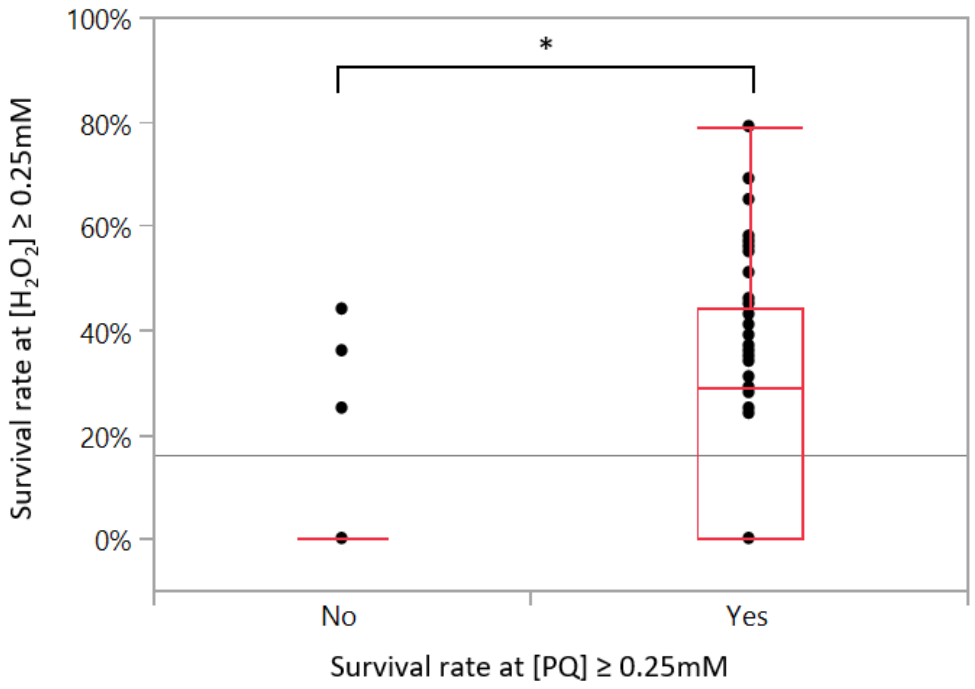


Figure 23: Survival rate of the 83 *C. jejuni* strains exposed at 0.25 mM H_2O_2 as a function of their survival to 0.25 mM PQ.

Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

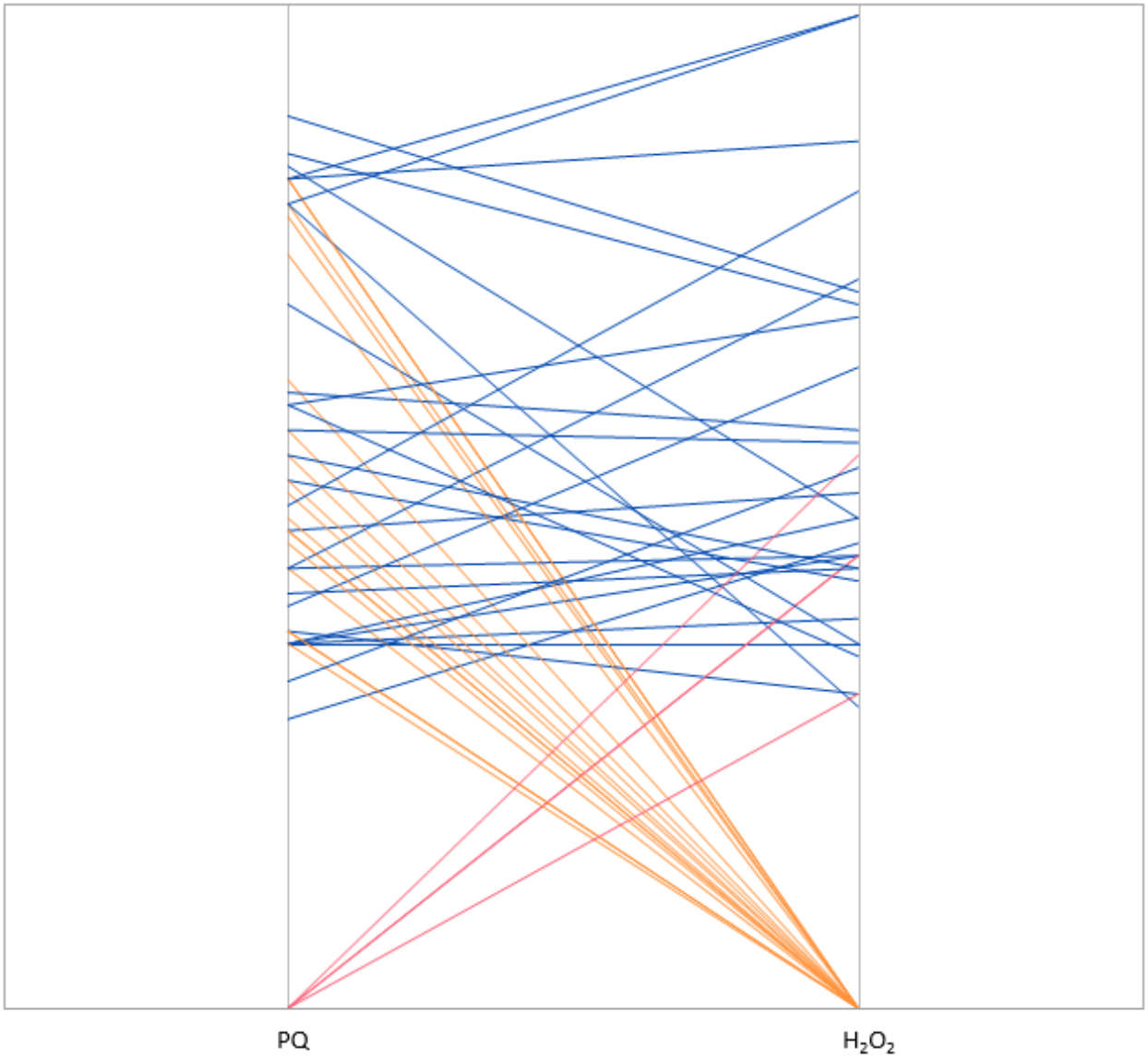


Figure 24: Parallel coordinates, relationship between survival at 0.25 mM PQ and H_2O_2.

Each line corresponds to a strain exposed to PQ (left y-axis) and to H_2O_2 (right y-axis). Blue line: strains surviving both stresses at a concentration of 0.25 mM (n = 27); orange line: strains surviving at 0.25 mM of PQ but not at 0.25 mM H_2O_2 (n = 20); red line: strains surviving at 0.25 mM H_2O_2 but not at 0.25 mM PQ (n = 4).

Consistent with this correlation and based on the calculation of the relative risk, it was highlighted that when a strain survives to a concentration equal to or above 0.25 mM PQ, it is six times more likely to survive to H_2O_2 at the same concentration (chi-squarest test, $p < 0.0001$) (Figure 25).

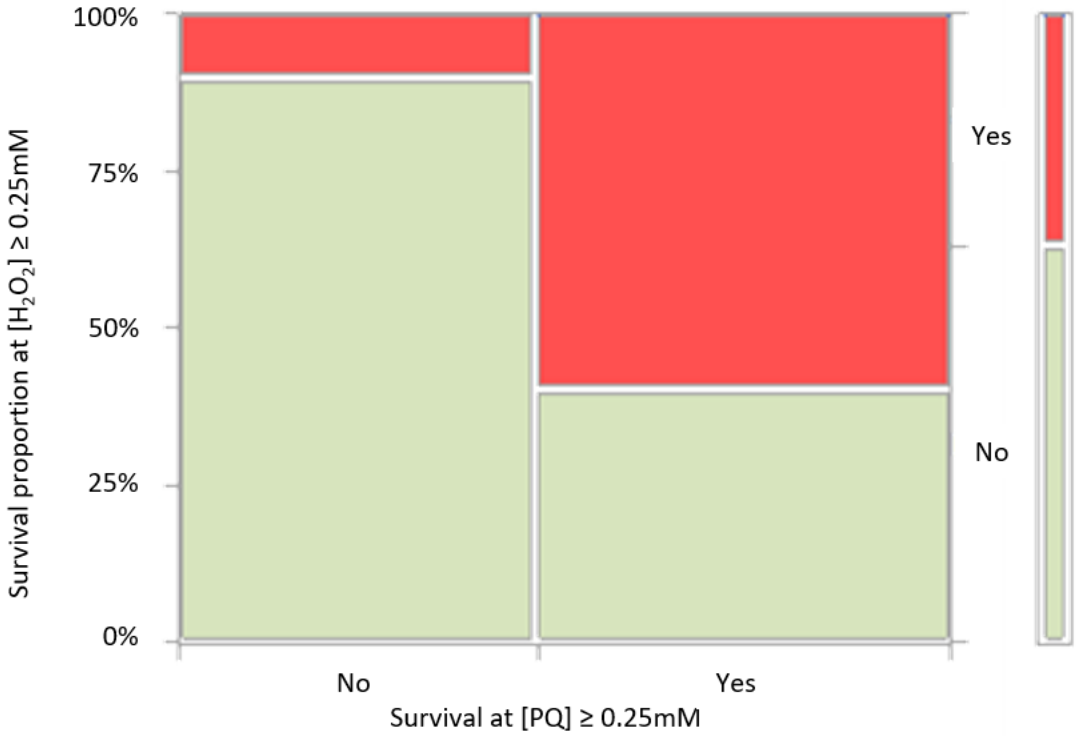


Figure 25: Correlation between survival to PQ (≥ 0.25 mM) and H_2O_2 (≥ 0.25 mM) stresses of 83 *C. jejuni* strains.

3.3. Acclimation to aerobic conditions

An acclimation test was selected to determine the capacity of multiplication of *C. jejuni* on solid media under AC, based on previous results obtained on the atypical strain Bf (Rodrigues et al., 2015). Gradual exposure to AC resulting in an increase in the number of colonies after three subcultures was validated for acclimation capability (Rodrigues et al., 2015). The results were expressed as follows: in the absence of colonies after the first subculture, the strains were defined as non-acclimated to AC (NAAC). In the case of growth only on charcoal-based selective medium after three subcultures but not on charcoal-blood free medium, an intermediate group of strains was classified as semi-acclimated to AC (SAAC). When colonies were detected after three subcultures on both media, the strains were classified as acclimated to AC (AAC). Surprisingly, the results indicate that 10 strains could acclimate to AC, irrespective to the

solid medium used, and 33 could acclimate after cultivation on selected medium. The other 40 strains did not show any acclimation capability (Table 11). Altogether, more than half of the strains (n = 43) were able to grow and multiply under aerobic conditions, while the others could not (Figure 26).

Table 11: Classification of 83 *C. jejuni* isolates submitted to gradual acclimation to aerobic condition (AC).

Level of acclimation	Number of strains
Non-acclimated to AC (NAAC)	40
Semi-acclimated to AC (SAAC)	33
Acclimated to AC (AAC)	10

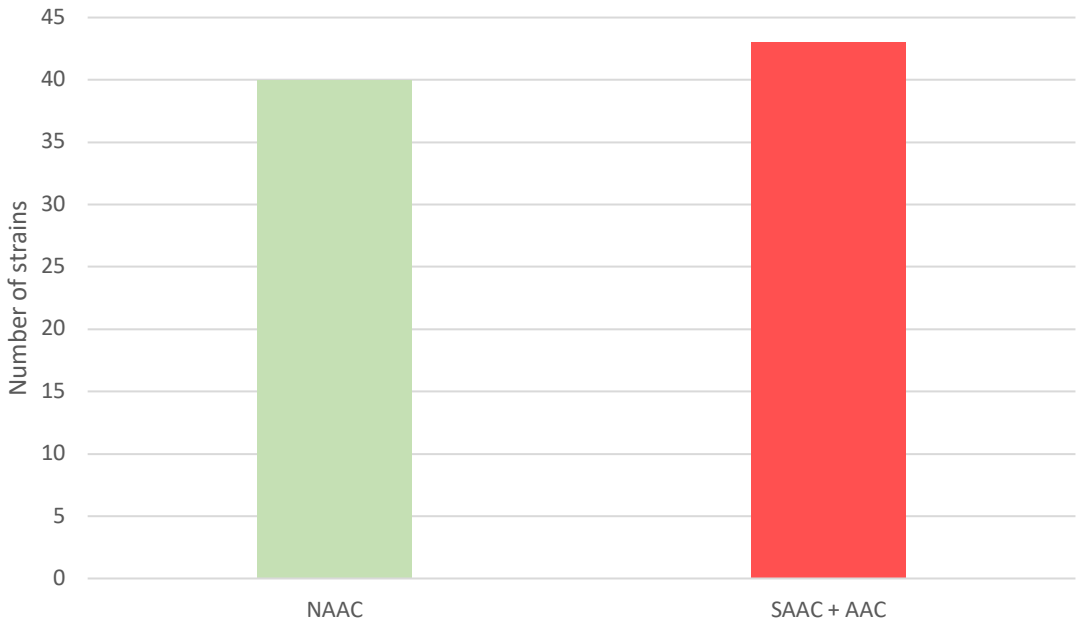


Figure 26: Distribution of non-acclimated (NAAC) and semi-acclimated or acclimated strains (SAAC and AAC) to aerobic conditions among the 83 *C. jejuni* strains.

To validate the acclimation ability, colonies grown on solid media after subcultures in AC were harvested. They were submitted to MALDI-TOF for identification and a minimum matching score of 2 was required for each *C. jejuni* strain, confirming the species. Part of these colonies were also stored at -80°C in the standard conditions. As a matter of fact, none of them were able to grow again when directly submitted to AC. Acclimation for these strains could be recovered only by using the same acclimation protocol with a first passage in microaerobic conditions. The acclimation capability was spread among the strains without any relation with the isolated sources (animal, human or environment).

For exploratory purposes, WGS data from 6 out of 10 strains before and after their acclimation to AC were analyzed with the cgMLST Oxford scheme (1,343 targets). According to the generated distance matrix, the four following strains were identical in alleles before and after acclimation to AC: Bf, Camp016, Camp018, and Camp036. Only one difference in allelic profile was recorded for Camp022 and Camp098 (Table 12). The changed target corresponded to CAMP046 in the cgMLST Oxford and it is related to the Cj0276 locus, also named *mreB*. This gene encodes for the MreB protein, a cell shape-determining protein. The two-point mutations at the nucleotide level are non-synonymous, leading to amino acid changes (Table 13).

	Bf	Bf-AAC	Camp016	Camp016-AAC	Camp018	Camp018-AAC	Camp022	Camp022-AAC	Camp036	Camp036-AAC	Camp098	Camp098-AAC
Bf	0											
Bf-AAC	0	0										
Camp016	1252	1230	0									
Camp016-AAC	1248	1237	0	0								
Camp018	1264	1241	2	1	0							
Camp018-AAC	1249	1237	2	1	0	0						
Camp022	1251	1230	10	10	11	11	0					
Camp022-AAC	1246	1235	11	11	12	12	1	0				
Camp036	1250	1225	11	11	13	13	8	9	0			
Camp036-AAC	1248	1237	12	12	13	13	8	9	0	0		
Camp098	1241	1216	938	932	944	933	937	930	933	929	0	
Camp098-AAC	1231	1216	929	934	938	934	929	931	928	932	1	0

Table 12: Distance matrix generated from cgMLST Oxford allele call table, for 6 *C. jejuni* strains, sequenced before and after their acclimation to aerobic conditions.

Table 13: Mutations detected after the cgMLST Oxford analysis, on 6 *C. jejuni* strains, before and after their acclimation to aerobic conditions.

Strain ID	Lineage	Cj0276 – *mreB* allele number		Mutations	AA substitution
		before	*after*		
Bf	UC	47	47	-	-
Camp016	A	21	21	-	-
Camp018	A	21	21	-	-
Camp022	A	21	822	G583A	Asp195Asn
Camp036	A	21	21	-	-
Camp098	UC	175	823	A224G	His76Arg

AA: amino acid; UC: unique combination

3.4. Adhesion to abiotic surfaces

Adhesion to an inert surface is measured after a 2-h incubation. After the plate reading, the results are expressed as BFI, and the ΔBFI is calculated. Among this panel, the ΔBFI average varied from 0.47 to 18.16 (Figure 27), the ΔBFI minimum being 0.00 and the ΔBFI maximum being 20.00. As the average ΔBFI was above seven, the closest integer value was chosen as the dividing line for categories ($\Delta BFI_{delimiting}$ = 8). Depending on the results obtained, the strains were classified in two significant distinct groups: high adhesion (ΔBFI ≥ 8) and low or no adhesion (ΔBFI < 8). Then, a total of 39 (47%) strains was classified with a high adhesion and 44 (53%) with a low or no adhesion (Figure 28). The positive control strain, Bf, displayed a high adhesion with a ΔBFI average of 17.47 (n = 12, standard deviation = 0.72), and the negative control strain, Camp052, developed very low adhesion with a ΔBFI average equals to 1.57 (n = 12, standard deviation = 1.57).

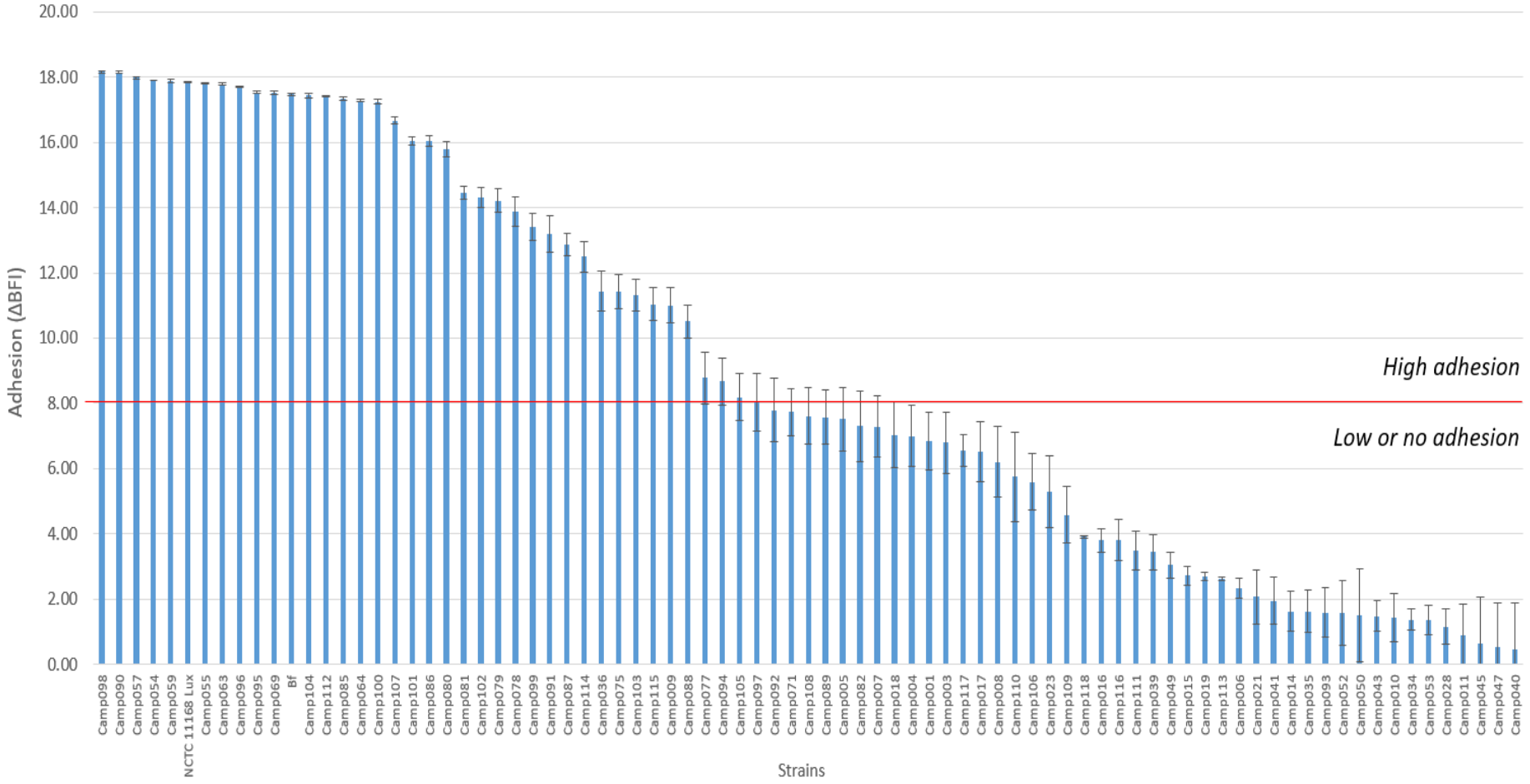


Figure 27: Adhesion of the 83 *C. jejuni* strains measured by the BioFilm Ring Test® after 2 h of contact to an inert surface in water using 10^9 ufc.mL^{-1} inoculum.

Error bars correspond to the relative standard deviation of the mean of three independent experiments. The red line separates strains with low or no adhesion (ΔBFI < 8) from the ones with high adhesion (ΔBFI ≥ 8). Positive and negative strain controls were used in each assay, Bf and Camp052, respectively.

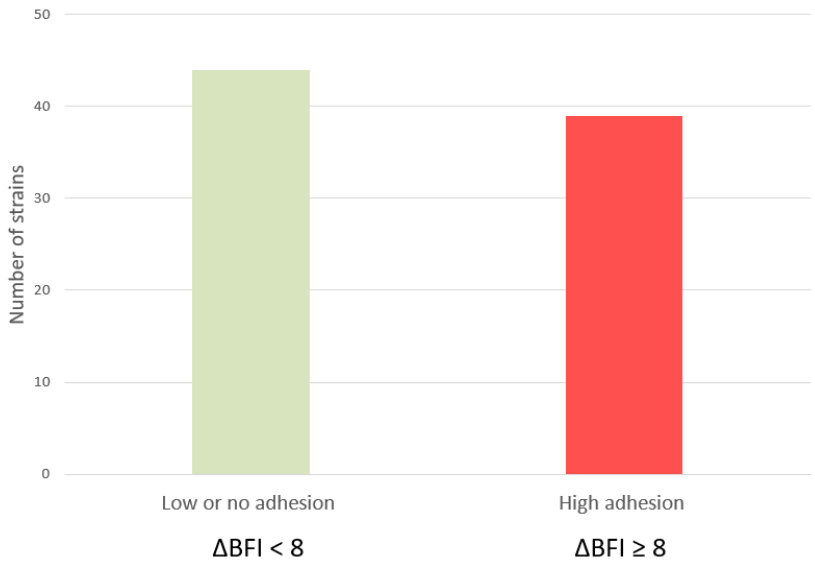


Figure 28: Classification of the 83 *C. jejuni* strains according to adhesion capability to abiotic surfaces.

3.5. Biofilm development

Given the ability of many strains to adhere to inert surfaces, one of the first steps required for biofilm development, it was interesting to go further and to determine if the strains displaying a high adhesion capacity could form biofilms as well. Although crystal violet is widely used to measure biofilm formation in diverse bacteria species (e.g., *Staphylococcus aureus, Listeria monocytogenes* or *Pseudomonas aeruginosa*), it is not well-adapted to characterize *Campylobacter* because of its low sensitivity (Sulaeman et al., 2010; Svensson et al., 2014; Grossman et al., 2021; Žiemytė et al., 2021). In contrast, the Biofilm Ring test® is a high-throughput method well designed to examine biofilm's early stage and development (e.g., *L. monocytogenes*, *E. coli*, *S. carnosus* and *S. xylosus*) (Chavant et al., 2007; Azeredo et al., 2017). Furthermore, with the need to characterize adhesion and biofilm development levels in parallel, the Biofilm Ring test® was the most relevant method to use in the present investigation. For that purpose, a specific protocol was built to assess the development of *Campylobacter* biofilm. It was based on protocols previously experimented for other bacteria by utilizing this magnetic beads technique (Chavant et al., 2007).

In order to ensure the bacterial multiplication associated with the biofilm formation process, a rich medium was selected to provide the necessary nutrients. Two parallel tests with the same bacterial inoculum (10^9 CFU.mL^{-1}) were carried out as follow: (i) a pre-incubation step in UPW for two hours at 42°C

in AC, followed by the addition of Brain Heart Infusion (BHI; BioFilm Control, France) mixed with magnetic beads; (ii) a direct incubation of the cells in tenfold BHI supplemented with magnetic beads. In both cases, the plates were read after an incubation time based on Turonova *et al.* procedure (2017) (Turonova et al., 2017). Very high values of the aggregation density were measured for all strains tested when they were cultivated directly in the rich medium BHI (tenfold diluted): saturation was rapidly reached which would explain the loss of variation in adhesion capacity and biofilm formation observed between strains. Whereas a pre-incubation step in UPW for two hours would select the growth of adherent bacteria only. Given the ability of many strains to adhere to a greater or lesser extent after two hours of incubation and the fact that free-living bacteria should not interact in the biofilm measurements, a supernatant removal step was initially tested before adding the rich medium. However, total removing of the supernatant resulted in many invalid technical replicates. These observations suggested that this latest action disrupts biofilm formation, potentially through the detachment of adherent bacteria after contact with the micropipette in the well bottom. A withdrawal of 190 µL gave better results in the reproduction of technical replicates and a pre-incubation in UPW had the advantage of collecting data for investigating on a potential link with the adhesion results, and on the changes in strain behavior. These parameters were therefore retained.

In order to finalize the protocol by selecting the best relevant incubation time to distinguish biofilm-producing from low and no-biofilm-producing strains, dynamics assays were performed. Based on Turonova *et al.* assays (2016), biofilm growth dynamics was explored at 20 h, 22 h, 24 h, 26 h, and 30 h on 24 strains of *C. jejuni* (Figure 29) (Turonova et al., 2016). As the results indicated a higher relevance between 22 and 24 h incubation time, the test was thus refined with more strains, and a reading at 22 h of incubation was selected. Finally, trials were carried out to identify two strains from the collection as quality controls (Bf for positive biofilm formation and Camp052 for negative biofilm formation). Both of them were used in each assay. As with the adhesion test, quality criteria were applied and technical replicates were considered valid when standard deviation between recorded values did not exceeded 1.5.

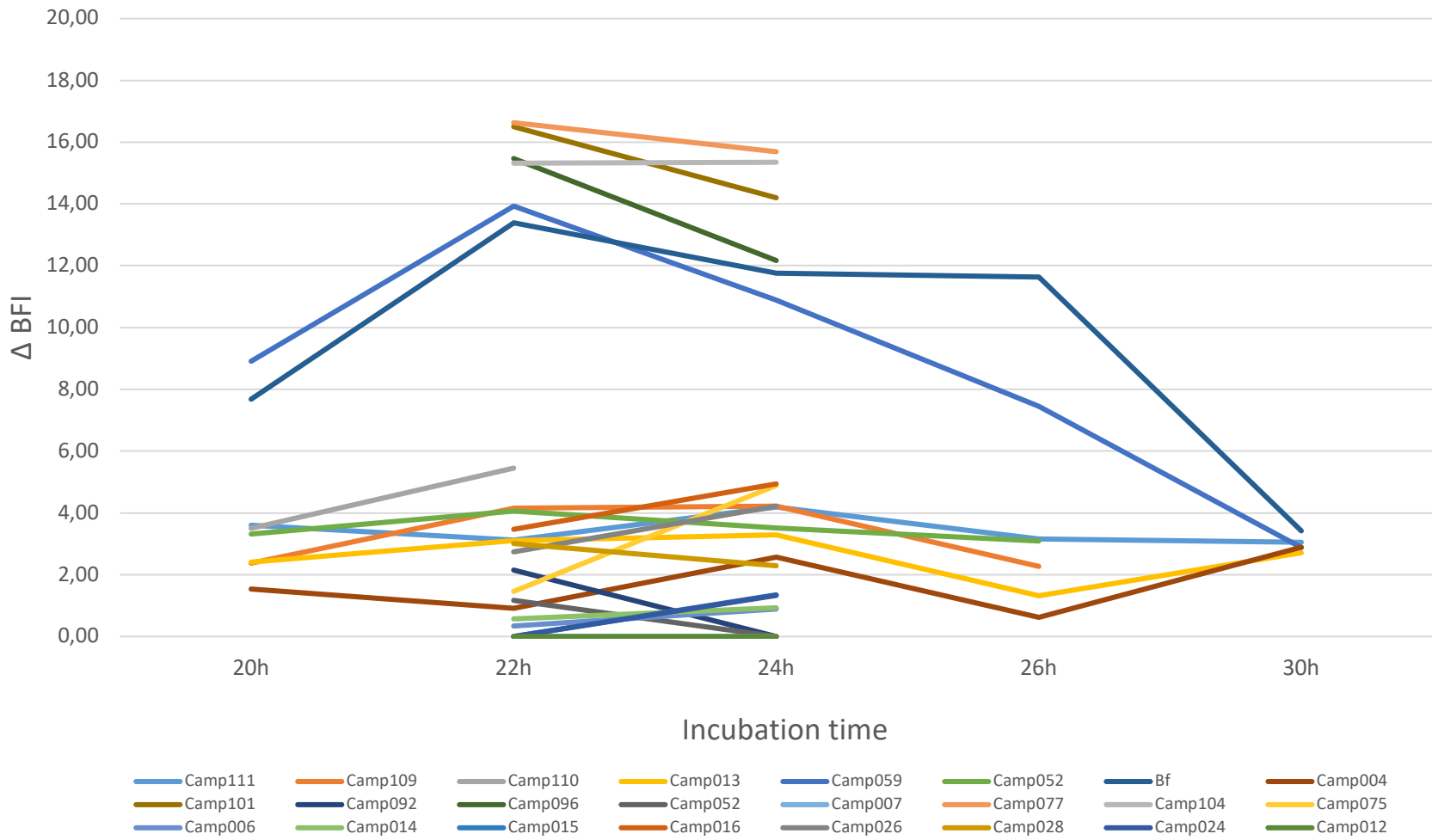


Figure 29: Biofilm growth dynamics exploration at 20 h, 22 h, 24 h, 26 h, and 30 h on 24 strains of *C. jejuni*.

After validation of the protocol, the entire collection of strains (n = 83) was analyzed for biofilm formation for each biological sample (Figure 30).

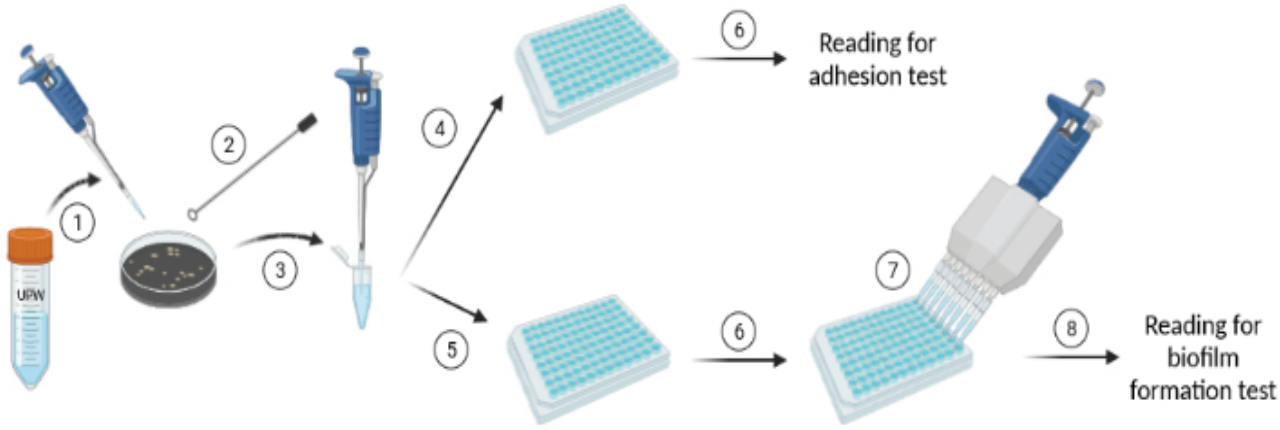


Figure 30: Biofilm protocol steps.

Steps 1, 2, and 3: culture harvested and resuspended in UPW. Steps 4 & 5: 200 µL of calibrated bacterial suspension mixed with the beads distributed in 96-well microtiter plates (three wells per biological sample) for adhesion and biofilm assays. Step 6: incubation at 42°C in AC for 2 h. Step 7: withdrawal of 190 µL of supernatant and replacement by tenfold diluted BHI mixed with the magnetic beads. Step 8: incubation at 42°C in AC for 20 h.

Biofilm formation was quantified after a total of 22-h incubation on the 83 strains. After the plate reading, the results were expressed as BFI, and the ΔBFI was calculated, exactly on the same principle than for adhesion assays. Among this panel, the ΔBFI average varied from 0.00 to 15.96 (Figure 31), the ΔBFI minimum being 0.00 and the ΔBFI maximum being 20.00. As the average ΔBFI was above seven, the closest integer value was chosen as the dividing line for categories ($\Delta BFI_{delimiting}$ = 8). Regarding the biofilm formation test, strains were classified in to two significant distinct groups: high biofilm formers (ΔBFI ≥ 8) and low or no biofilm formers (ΔBFI < 8). A total of 27 (33%) strains exhibited significant biofilm formation, while 56 (67%) displayed no or low biofilm development (Figure 32). The positive control strain, Bf, displayed a high adhesion with a ΔBFI average of 13.42 (n = 13, standard deviation = 1.54), and the negative control strain, Camp052, developed very low adhesion with a ΔBFI average equals to 1.05 (n = 13, standard deviation = 0.91) (Figure 31).

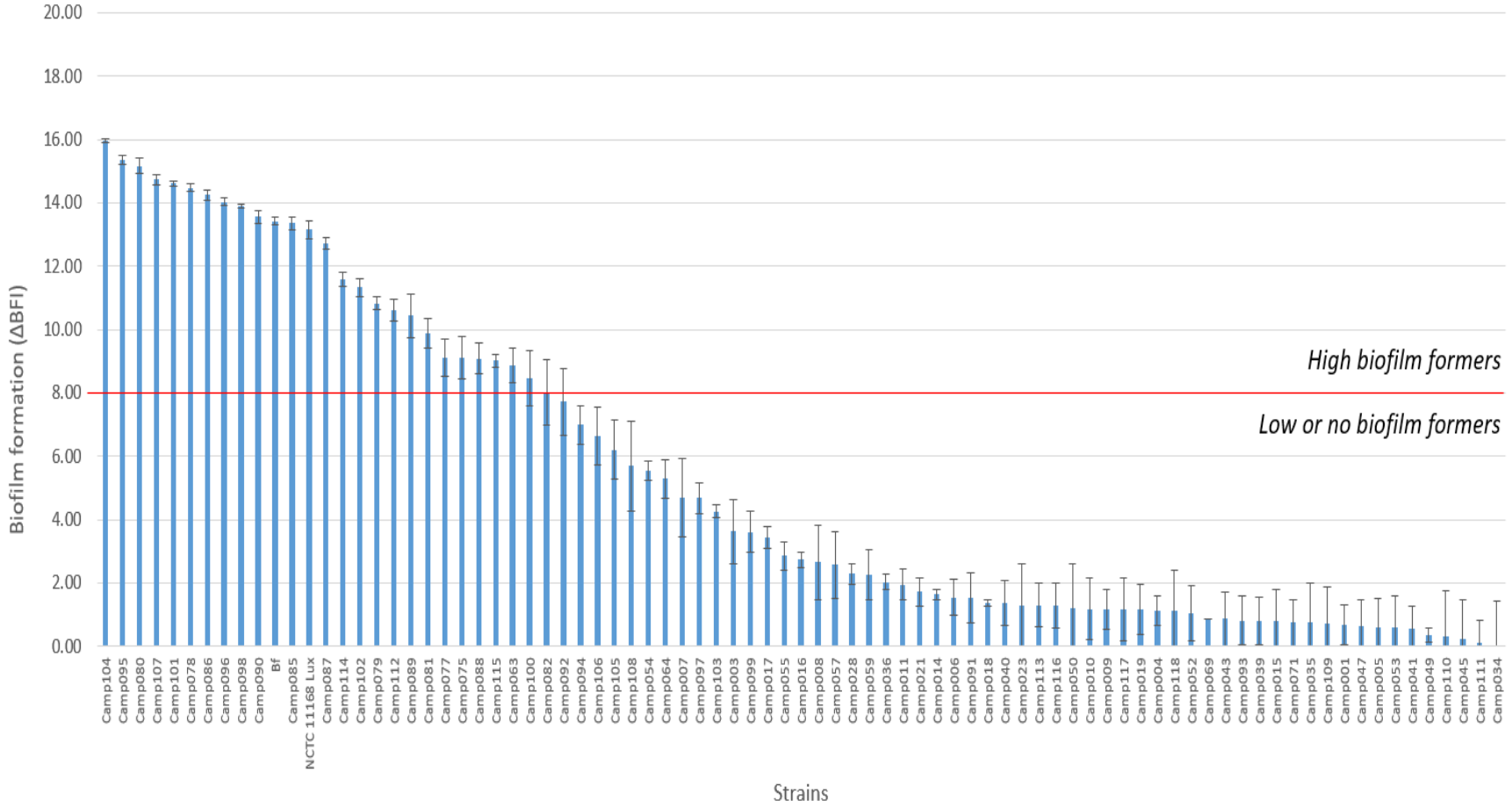


Figure 31: Biofilm formation of the 83 *C. jejuni* strains measured by the BioFilm Ring Test® after 22 h of incubation in 10X diluted BHI from adhered cells.

Error bars correspond to the relative standard deviation of the mean of three independent experiments. The red line separates strains with low or no biofilm formers (ΔBFI < 8) from high biofilm formers (ΔBFI ≥ 8). Positive and negative strain controls were used in each assay, Bf and Camp052, respectively.

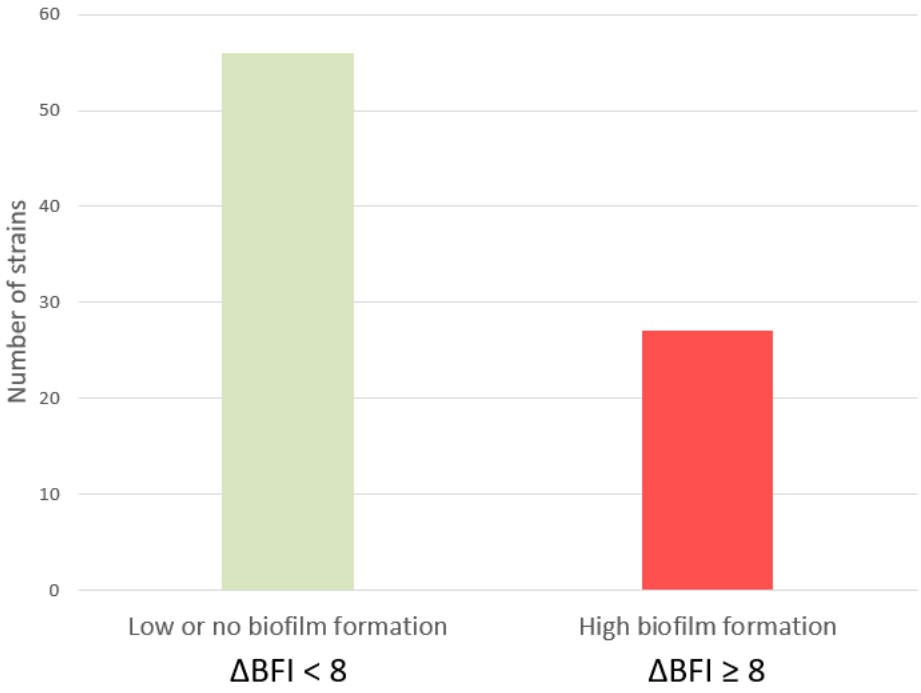


Figure 32: Classification of the 83 *C. jejuni* strains according biofilm formation ability.

3.6. Correlation between adhesion and biofilm formation

Statistical analyses were based on qualitative variables for the chi-squared test, the level of adhesion, and biofilm formation (high adhesion or low/no adhesion, high biofilm formation or low/no biofilm formation). They were performed on quantitative variables for the ANOVA test, the proportion of biofilm-forming strains, and the ΔBFI. Most of the strains were able to form a biofilm when they exhibited a high adhesion (n = 25), and strains (n = 42) with no or weak adhesion could not form biofilm. The ability to display a high adhesion was significantly associated with the high formation of biofilm (ANOVA, $p < 0.0001$) (Figure 33).

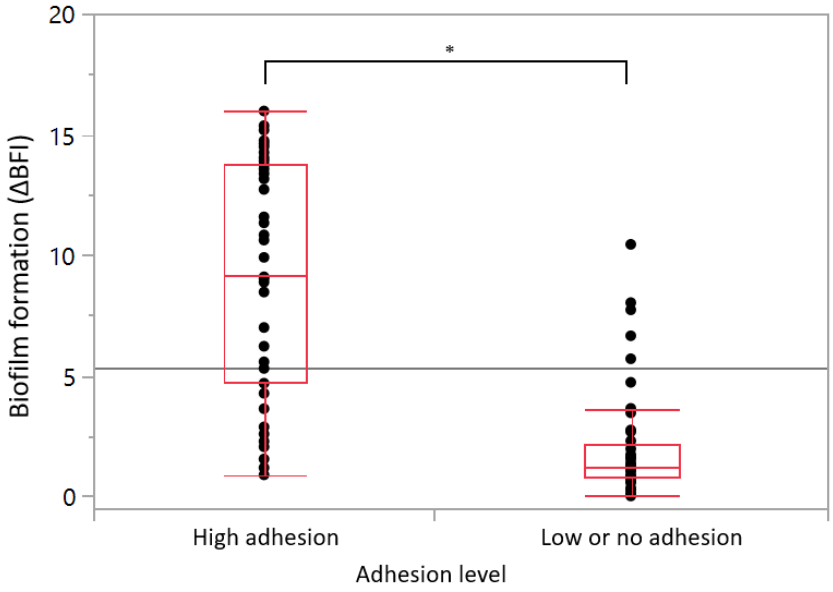


Figure 33: Distribution of the 83 *C. jejuni* strains biofilm formation according to their adhesion capability. Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

It has also been highlighted that when a strain has a strong adhesion, it is 14 times more likely to develop a biofilm (chi-squarest test, $p < 0.0001$) (Figure 34). Interestingly, 14 strains were not able to form a biofilm, although they have a high adhesion.

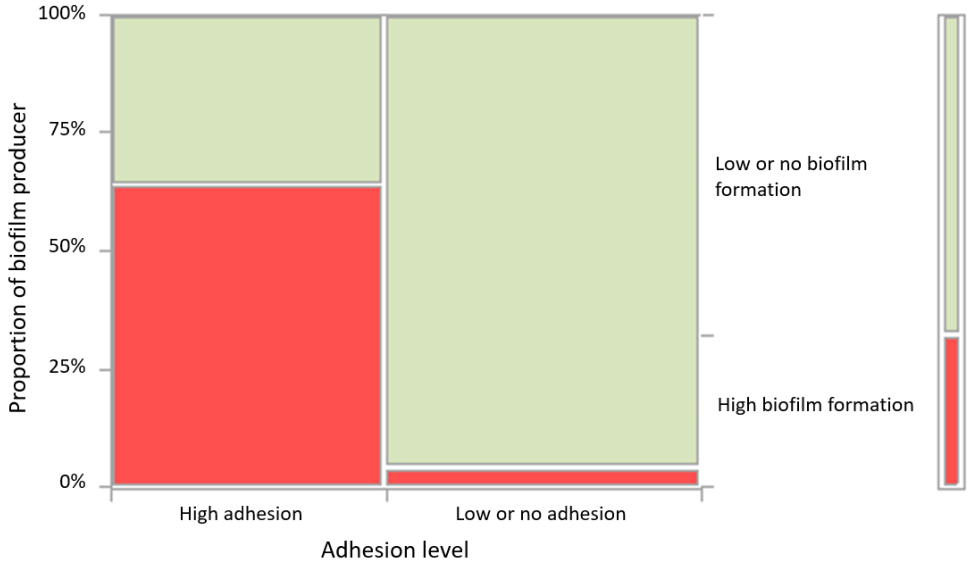


Figure 34: Global correlation between adhesion capability and biofilm producers.

3.7. Additional inter-test correlations

Unexpectedly, acclimation to aerobic conditions shows no significant correlation with oxidative stresses, neither adhesion capability nor biofilm formation. Furthermore, there was no correlation between hyperoxide stress, adhesion to an inert surface, and biofilm formation. In addition, only a weak correlation was established between superoxide stress and the ability of strains to adhere to abiotic surfaces (ANOVA, $p = 0.0454$) (Figure 35).

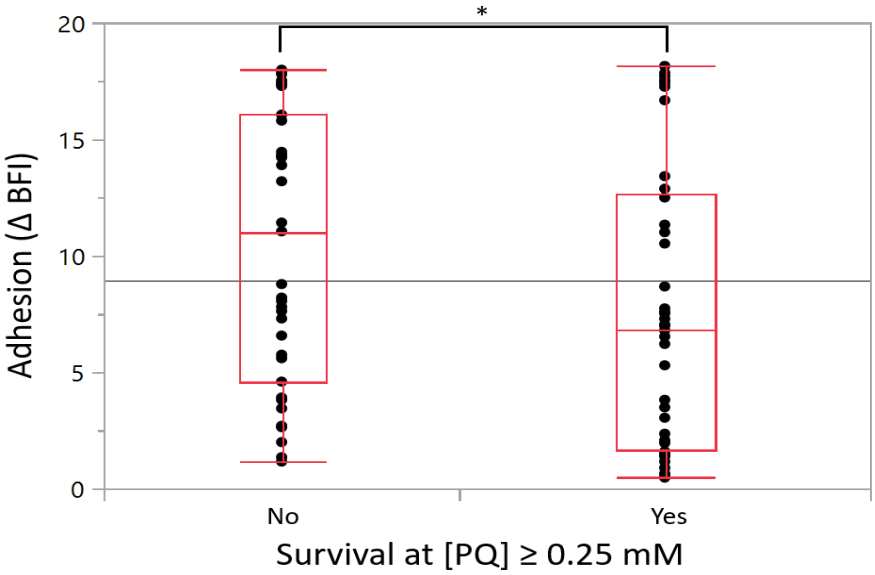


Figure 35: Distribution of the survival rate of the 83 *C. jejuni* strains exposed to at most 0.25 mM PQ according to their adhesion capacity.

Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

4. Discussion

Our previous study highlighted the presence of monomorphic lineages regularly infecting humans with a broad distribution over time and sources, suggesting persistence in reservoirs and the environment. It points out the possible long-term existence of stable lineages appearing based on the concordance of the different typing schemes, as mentioned in chapter II. Bacteria may display different phenotypes, resulting from their genomic plasticity, allowing them to colonize other hosts or to spread in the environment. This work aimed to investigate the phenotypical traits that could contribute to the emergence and persistence of *C. jejuni*. To characterize phenotypes related to transmission and persistence of *C. jejuni*, three different tests were selected to cover the adaptation potentiality of *C. jejuni* based on previous studies on the higher risks of transmission. This potentiality was investigated through *in vitro* assays in controlled conditions revealing susceptibility to oxidative stresses, acclimation ability to aerobic conditions, adhesion capability to inert surface and biofilm development.

A collection of 83 *C. jejuni* was set up. It was composed of strains isolated in Luxembourg from various sources (human, mammals, poultry, and environment), different spreading profiles (endemic, epidemic and sporadic), and defined representative genomic lineages. The total number of strains (n = 83) was defined to handle all the phenotypical tests including replicates. To be consistent and to limit biological variation, the same inoculum was used to perform all the tests except for biofilm, which required the development of a new protocol. However, robustness was ensured using positive and negative biofilm former controls for each assay.

Several studies have investigated how to score the impact of oxidative stresses on bacterial survival by implementing different techniques. Oh *et al.* (2015) grew bacterial cultures under aerobic conditions and then measured the level of ROS formed, while Mouftah *et al.* (2021) used peracetic acid for their chemical decontamination stress survival test, which is known to decompose to H_2O_2 and acetic acid (Oh et al., 2015b; Mouftah et al., 2021). Dai *et al.* (2017) used PQ and H_2O_2 to detect specific spontaneous mutants and point mutations conferring resistance (Dai et al., 2017). These molecules were also selected by Hwang *et al.* (2011) to analyze the regulation of oxidative stress response by CosR (*Campylobacter* oxidative stress regulator; Cj03555c) and by Rodrigues *et al.* (2015) to determine the expression of the *mreB* gene maintaining the bacillary morphology (Hwang et al., 2011; Rodrigues et al., 2015). In our study, these molecules (PQ and H_2O_2) were used as prooxidant to generate oxidative stresses on *C. jejuni*. PQ simulates the superoxide stress while H_2O_2 focuses on the hyperoxide stress. These two stresses were investigated as *C. jejuni* possesses a broad range of enzymes involved in oxidative stresses

defense, and different detoxification mechanisms are involved in PQ and H_2O_2 degradation. The unique superoxide dismutase (SOD) in *C. jejuni* is an enzyme encoded by the *sodB* gene that converts superoxide radicals from PQ to hydrogen peroxide and dioxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$) (Pesci et al., 1994; Atack and Kelly, 2009). Then, the unique catalase KatA, encoded by the *katA* gene, splits hydrogen peroxide into water and dioxygen ($2H_2O_2 \rightarrow 2H_2O + O_2$) (Grant and Park, 1995; Day et al., 2000; Garénaux et al., 2008). Other unneglectable enzymes, the alkyl hydroperoxide reductase (AhpC) and the thiolperoxidase (Tpx), are also involved in this H_2O_2 detoxification mechanism by converting peroxides in alcohols (Atack et al., 2008; Atack and Kelly, 2009).

In our study, the correlation between H_2O_2 and PQ survival at a concentration of 0.25 mM was demonstrated: 32.5% (n = 27) of the strains were resistant to both stresses. These results are consistent with Rodrigues *et al.* (2015) work performed on the *C. jejuni* Bf strain, indicating a lower susceptibility to both oxidant agents (Rodrigues et al., 2015). CosR was found to negatively control SodB and positively regulate AhpC and KatA (Hwang et al., 2011, 2012). However, in the presence of PQ, the CosR protein expression level is reduced (Hwang et al., 2011). This could dysregulate SodB and lead to an increase in its activity and thus to an accumulation of H_2O_2, increasing the sensitivity of the strains and decreasing their survival, as described for *E. coli* (Scott et al., 1987). This could explain why, at the same concentration of 0.25 mM tested, some strains did not survive to oxidative stress generated by PQ but managed to survive to H_2O_2 assays suggesting that H_2O_2 accumulation is possibly higher in the PQ assay than in the H_2O_2 one, carried out separately. On the contrary, a better survival in PQ assays at the same concentration, could result in an upregulation of KatA activity (Garénaux et al., 2008), actively detoxifying the H_2O_2 accumulated in excess.

C. jejuni is a strict microaerophilic pathogen requiring a low concentration of dioxygen (~ 5-6%) for optimal growth, and the main stress to face to in the extra-intestinal environment is likely the atmospheric concentration of O_2 (Kaakoush et al., 2007; Macé et al., 2015). Reduced susceptibility or acclimation ability to aerobic conditions would confer greater environmental persistence, thus increasing the risk of transmission between prospective hosts. As described by Oh *et al.* (2018), aerotolerance contributes to the survival of *C. jejuni* in chilled chickens and its prevalence in human clinical cases (Oh et al., 2018). Numerous other studies have investigated *C. jejuni* aerotolerance capacity, demonstrating the high prevalence of hyper-aerotolerant strains in poultry too (raw chicken and duck meat) (Kim et al., 2019). Likewise, other studies investigated the mechanism of regulation of the aerotolerance (Baillon et al., 1999; Atack et al., 2008; Handley et al., 2015). For instance, through transcriptomic and proteomic

assays, it was previously highlighted that many proteins involved in host colonization (e.g., PorA and CadF) were more abundant at lower oxygen availability (1.8% O_2) while a coordinated response of oxidative stress protection enzymes (e.g., SodB, AhpC, Tpx, and TrB) and Fe-S cluster biogenesis proteins was observed in oxidative stress conditions (i.e., increased level of O_2 from 5% to 17.5%) (Guccione et al., 2017). Rodrigues *et al.* (2016) have also performed transcriptomic and proteomic analyses on the Bf strain, and they noticed higher transcript levels for Tpx and SodB in AC than in MAC (Rodrigues et al., 2016). However, most of the studies were based on a survival detection under harmful conditions. Before starting my thesis in March 2018, only one strain of *C. jejuni* (Rodrigues et al., 2015) and one of *C. coli* (O'Kane and Connerton, 2017) have been described in the literature as being able to acclimate and multiply in AC. The growth was obtained after several passages in solid media in harmful conditions. In order to figure out if this unique fitness concerns only a few strains, the collection of *C. jejuni* was screened for aerobic acclimation in this study. Surprisingly, the results indicate that more than half of the strains could acclimate to AC. Among them, approximately 10% were even able to grow in Columbia agar, a nutritive medium without any oxygen-reducer. For most of the isolates, aerobic growth was low during the first sub-culture but significantly enhanced after the two subsequent passages. With the implementation of an intermediate step, growth on selective charcoal-based medium acting as an oxygen-reducer, we were able to segregate between the strains with higher acclimation capability (AAC) from those with lower acclimation ability (SAAC). A minimum of three subcultures was required to ensure the reproducibility of the data. Very recently, the same conclusions were observed on several strains of *C. jejuni* able to acclimate to AC with a reproducibility of the results obtained after three passages (Shagieva et al., 2021). The authors focused on the study of *C. jejuni* harvested from wastewater and intracellular growth preservation into amoebas. Our study indicates that this capability to acclimate to AC is not related to the isolated sources as strains from clinical, animal or environment displayed this ability. Noticeably, in our study, the acclimated colonies did not show any capability to growth again in AC directly after storage at - 80°C. The phenomenon of acclimation for those colonies could only be obtained after growing in MAC and at least three passages under AC. This indicates that this acclimation capability is not acquired and did not result from stable genetic modifications. Rodrigues *et al.* (2015) highlighted that the *C. jejuni* Bf strain, acclimated to aerobic conditions, was less susceptible to oxidative stress than the other strains unable to multiply under AC (Rodrigues et al., 2015). On our larger scale collection, our results did not show any significant correlation between resistance to oxidative stresses and aerobic acclimation levels.

Several new methodologies have been developed recently to study biofilms, including their cultivation and characterization. They involve different approaches to evaluate the adhesion level and

techniques to measure biomass, viability, and matrix composition. Microtiter plates and the Biofilm Ring test® are used to screen for the formation capacity and test anti-biofilm molecules, while crystal violet staining is instead employed to measure the biomass (Azeredo et al., 2017). However, although the crystal violet test is widely used to measure biofilm formation in multiple bacterial species, it is not adapted to *Campylobacter* detection because of a lower sensitivity (Sulaeman et al., 2010; Svensson et al., 2014). In contrast, the Biofilm Ring test® is a high-throughput, easy to handle method without washing, fixation, nor staining, and well designed to examine the early stage of biofilm formation (Chavant et al., 2007; Azeredo et al., 2017). A study comparing bacterial adhesion with the BioFilm Control and reverse ELISA methods has validated the use of the BioFilm Ring test® for *Campylobacter* (Sulaeman et al., 2010). Briefly, this test is based on the immobilization of magnetic beads by attached cells. If cells adhere to the inert surface, they trap the beads, which are less detectable (Chavant et al., 2007). Due to the large number of *C. jejuni* strains to be analysed and the need to characterize their levels of adhesion and biofilm development in parallel, the Biofilm Ring test® was the most relevant method to use in the current experiments. To that purpose, a specific protocol was developed for *Campylobacter* biofilm development based on the protocols previously established with other bacteria using this technique (Chavant et al., 2007).

The results of adhesion and biofilm formation tests indicated a wide diversity in adaptive responses among *C. jejuni* strains going from no adhesion to high adhesion and no biofilm producer to high biofilm-producing strains. Growing in AC was a factor of success for biofilm formation. This result is in accordance with previous studies on *C. jejuni* (Gunther and Chen, 2009; Sulaeman et al., 2010; Turonova et al., 2015; Teh et al., 2019). By screening a large collection of strains, a strong correlation could be established between adhesion to abiotic surfaces and biofilm formation. Indeed, a highly adherent strain is 14 times more likely to develop a biofilm. The same correlation was reported by Faria *et al.* (2021), who found a significant correlation between both steps for cyanobacteria (Faria et al., 2021). Previously, adhesion analyses to abiotic surfaces by *L. monocytogenes* showed a diversity in the capability to attach to inert surface, however all the strains were able to adhere, indicating a greater diversity for *C. jejuni* (Tresse et al., 2007). In our study, some strains could not attach to any surface, which is in accordance with previous results on *C. jejuni* or *C. coli* (Sulaeman et al., 2010). Only two strains were able to form a biofilm while no adhesion was observed for them. One hypothesis is that these strains have a delayed adhesion that could not be measured after two hours of incubation. This phenomenon has already been observed on 6 *C. jejuni* strains (including the Bf strain) by Bronnec *et al.* (2016) (Bronnec et al., 2016). Furthermore, 14 strains were significantly able to adhere firmly but not to produce a strong biofilm. This

behaviour was previously described for the strain NCTC 11168, qualified as the poorer biofilm-forming strain, compared to the strain 81-176 (Turonova et al., 2015). Using confocal microscopy observations, these authors observed a dense biofilm showing layers of attached cells without any pores and channels to ensure nutrient and gas exchanges, which consequently hindered the survival of the biofilm. In that case, the authors have hypothesized that the biofilm could not enter in a maturation phase. A two-component regulator, CosR, or enzymes, such as AhpC, appear to play a role in this biofilm maturation step (Oh and Jeon, 2014; Turonova et al., 2015). *C. jejuni* attachment to an inert surface can also be promoted by the presence of a pre-established biofilm (Hanning et al., 2008). Indeed, the biofilm formation of *Campylobacter* strains was shown to be significantly enhanced in the presence of *S. aureus* or *P. aeruginosa* cells already attached (Teh et al., 2019; Karki et al., 2021). Another hypothesis is that these strains may be part of the biofilm only as a secondary colonizer, which would explain their high adhesion capacity and low biofilm formation ability. Our study indicates that several strains seem to be able to initiate biofilm by themselves.

The mechanisms of adaptive strategies adopted by *C. jejuni* to survive in the environment remain elusive. Further studies are required for a better understanding of the biological mechanisms underlying its atypical capacity to adapt to and multiply in aerobic conditions, for instance. Except the intra-test correlation, no correlation between the phenotypical responses was established. Therefore, we investigated whether the recurrent lineages or sporadic strains have specific phenotypic features to explain the persistence over time.

Chapter IV: Relationship between recurring genomic lineages and phenotypes related to persistence and transmission

Partially as in: Nennig M, Clément A, Longueval E, Bernardi T, Ragimbeau C and Tresse O. New insights on phenotypic traits associated with *Campylobacter jejuni* recurring lineages identified in human infections in Luxembourg. *Manuscript in preparation*.

Introduction

Persistence in reservoirs, sources and/or the environment could be linked to specific phenotypic traits contributing to the survival abilities outside the hosts of these lineages. Phenotypic traits that could explain the transmission and the persistence of bacteria in the environment are numerous. However, considering the fastidious nature of *C. jejuni*, biological advantages that could be involved are reduced. The genetic equipment of *C. jejuni* appears as limited when compared to other pathogens. For instance, the lack of RpoS factor could limit responses to general stress (Parkhill et al., 2000). Carbon source assimilation is also a factor limiting the adaptation capabilities of *C. jejuni*. Although the presence of a genomic island was identified in some strains to challenge enteric bacteria by uptaking and utilizing L-fucose (Stahl et al., 2012) and 1.7% of over 6,000 *C. jejuni* strains were able to restore the glycolysis via Entner-Doudoroff pathway (Vegge et al., 2016), most of the strains remain asaccharolytic, which reduces niches for multiplication. The uptake of amino acids, which is the main carbon source in *C. jejuni,* is correlated to osmoregulation. Although some adaptations to hyper- or low-osmotic environments have been described for *C. jejuni*, they are temperature-dependent and remain limited (Burgess et al., 2016). In addition, *C. jejuni* is sensitive to desiccation. The minimum a_w for growing *Campylobacter* has been defined at 0.987 with an optimum at 0.997 (Line, 2006). Bacterial fitness can also be explored at another level. Recent studies investigated on the frequency of AMR within specific phylogenetic lineages. They provided evidence of clonal expansion of particular fluoroquinolone-resistant *C. jejuni* lineages (e.g., CC ST-21, or CC ST-464), suggesting that the point mutation in the gyrase, conferring the AMR, grants a robust selective advantage that could explain persistence over time (Han et al., 2012; Wimalarathna et al., 2013; Kovač et al., 2014; Cha et al., 2016).

Relatively little is known about the mechanisms of pathogenicity in *C. jejuni*, but some virulence factors have been identified. They are mainly involved in its capability to interact with, to adhere to, and to invade host cells (Lugert et al., 2015). In addition, they were not related to the persistence or transmission of *C. jejuni*. For instance, virulence could not be associated with the cross-contamination of *C. jejuni* or *C. coli* from naturally contaminated chicken legs to other foodstuff through a cutting board (Guyard-Nicodème et al., 2013). Similarly, the high prevalence of *C. jejuni* strains, harboring multiple virulence genes, was highlighted at the farm level but without any correlation with their persistence (Rossler et al., 2020). Consequently, virulence factors were not inspected in this study.

Phenotypes related to oxidative stress, aerobic conditions, or survival on surfaces are the ones that could contribute to transmission and persistence of *Campylobacter*. However, these adaptive capabilities were described only on a few strains, focusing mainly on well-studying strains (Turonova et al., 2015; Oh et al., 2016; Park et al., 2021). Their frequency and distribution among the genetic population are elusive. These specific phenotypic features, including antimicrobial susceptibility, were investigated in our study, on 83 NGS-characterized strains of *C. jejuni*, being representative of isolates from multi-hosts over 13 years in Luxembourg. In the previous chapter, we demonstrated that strains could be classified according to their abilities to respond to oxidative stresses, to acclimate to aerobic conditions, to be able to adhere to abiotic surfaces and to develop biofilms. A correlation was found in response to the hyperoxide and superoxide stresses which could be explained by the overlapping of enzymes required to scavenge accumulated ROS. In parallel, a correlation was observed between adhesion ability and biofilm formation, which was explained by the adhesion step required to initiate biofilms. Surprisingly, no significant correlation was stated between oxidative stress responses, acclimation to aerobic conditions and biofilm formation capability. This latter result indicates that the cellular and molecular mechanisms underlying these phenotypes are different. In this chapter, we statistically explore whether each phenotype could be correlated to epidemic profile and genomic lineages. When a correlation was found, a functional genomic analysis was applied to identify which loci could be involved in this correlation.

1. Material and methods

1.1. Genotyping data of *C. jejuni* isolates

WGS data associated with this strain panel were collected from data collected in Chapter II. DNA extraction, library preparation, sequencing analyses, and quality control were performed as previously described in Chapter II. Additional raw reads have been uploaded to ENA (*in progress*).

The typing scheme for WGS analysis implemented in SeqSphere+ v6.1 (Ridom GmbH, Münster, Germany) was used as previously described in chapter II. The graphical representation of the phylogenetic analyses was constructed through a UPGMA tree based on the cgMLST allelic profiles. The pairwise ignoring missing values was used as the setting.

1.2. Antimicrobial resistance

Predicted AMR patterns were established using the analysis of the *gyrA* alleles as previously described by Ragimbeau and colleagues (Ragimbeau et al., 2014).

1.3. Statistical analyses

Statistical analyses included the comparison of groups of strains, according to the survival to oxidative stresses (H_2O_2 and PQ), the acclimation to aerobic conditions, the adhesion capacity, the biofilm formation ability, the antimicrobial susceptibility, the recurrence, and the belonging to a particular genomic lineage. The results were analysed with JMP v.15 software (SAS Institute Inc., North Carolina, USA), and Microsoft® Excel® 2016 (v.16.0.5239.1001), using the chi-squared test for qualitative variables and variance analysis (ANOVA) for quantitative variables. The significance level was determined at 95%, $p<0.05$ considered as significant. When multiple comparisons were performed, the confidence levels for each comparison performed had to be higher to be validated, so that the result of the multiple comparisons meets the 95% confidence level. For this purpose, the Tukey-Kramer method was used to keep the alpha risk at 5%. Distributions were displayed using box and whisker diagrams for graphical representation.

1.4. *In silico* identification of the unique targets from the pan genome

The specific targets belonging to the four different lineages were previously determined using the wgMLST INNUENDO typing scheme in chapter II. As targets may be only a part of a gene, their presence, location and organization were explored using the Platform MicroScope (https://mage.genoscope.cns.fr/) (Vallenet et al., 2017, 2020). Thanks to the possible manual annotation of strains uploaded on this platform, protein identification was performed whenever it was possible. This analysis was completed and the biological functions of the detected protein were inferred using KEGG and UniProt (Kanehisa et al., 2016; The UniProt Consortium, 2021).

1.5. *In silico* identification of the T6SS core components

The presence of the type VI secretion system (T6SS) core components was investigated in the recurrent strains of our collection. As a fully assembled T6SS is composed of a minimal of 13 core components, the list of genes of interest was extracted from Zoued *et al.* (2014): *tssJ*, *tssK*, *tssL*, *tssM*, *tssA*, *tssB*, *tssC*, *hcp*, *tssE*, *tssF*, *tssG*, *clpV*, and *vgrG* (Zoued et al., 2014). Nucleotide and amino acid sequences of the genes were collected from the NCBI RefSeq genome database, from the *C. jejuni* 488 strain (Liaw et al., 2019), in order to confirm their presence or absence in all the recurrent strains. BlastN and blastP were performed using the Platform MicroScope for the first screening on the strains. Geneious® v.11.1.3 (https://www.geneious.com/) was used to map specific sequences against whole genome of the strains, and BioEdit v.7.0.5.3 (www.mbio.ncsu.edu/BioEdit/BioEdit.html) was used to align the target sequences to visualize potential mutations.

2. Results

2.1. Genetic diversity of the strain collection tested

In chapter II, the cgMLST typing scheme was validated to classify the strains in the collection used in this study. Using this expertise to perform phylogenetic analyses, the strains could be classified in different recurring genomic lineages: 22, 10, 8, and 12 strains belonged to lineages A, B, C, and D, respectively. In addition, 29 strains from clinical and environmental sources displayed a unique genomic profile (Unique Combination, UC) as well as NCTC 11168 and Bf strains (Figure 36). According to the distance matrix (Appendix B), strains belonging to lineage A had at least 428 allele differences (AD) from the other strains.

Strains belonging to lineages B, C, and D, had at least 305, 1019, and 912 AD from the other strains. These results corroborated that the four lineages were genetically distant from each other. Furthermore, strains, classified as human UC, presented at least 109 AD with other strains, except Camp015 and Camp106, which are distant from 12 AD only. As the cluster alert implemented for the cgMLST Oxford was at 11 AD, these two strains were therefore considered as distinct profiles. The environmental strains with unique combinations had at least 642 AD with the other strains. The reference strain, NCTC 11168, and the Bf strain were distant from the others at least at 247 and 1,177 AD, respectively.

As described by Dingle *et al.* (2001), it is possible to classify the strains into groups of related isolates, namely the clonal complexes (CC), based on the MSLT data (Dingle et al., 2001). Thus, the lineages A and D belong to the CC ST-21 and are considered as host-generalist lineages (Figure 36). The lineages B and C could be classified in two different host-specialist lineages: the CC ST-257 and the CC ST-464, respectively, as described by Sheppard et al. (2014) (Figure 36) (Sheppard et al., 2014).

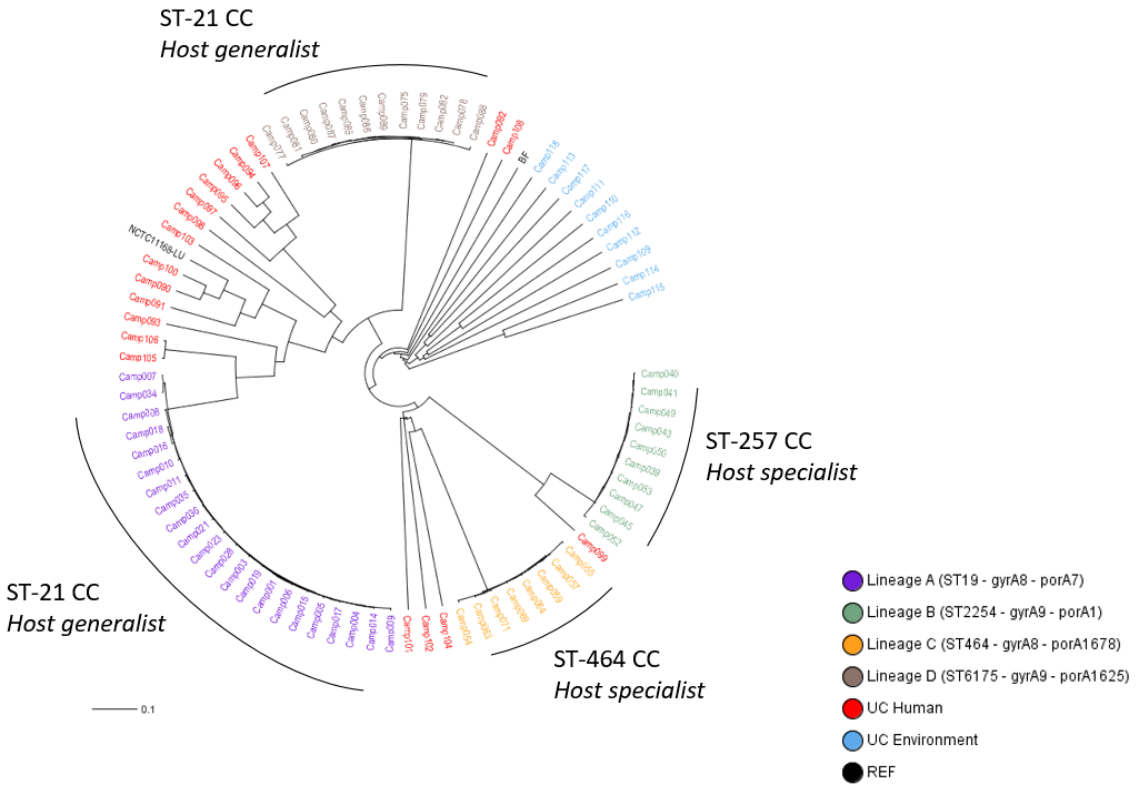


Figure 36: UPGMA phylogenetic tree for 83 *C. jejuni* isolates based on the Oxford cgMLST allelic profiles (1,343 loci).

2.2. Correlation between recurrence pattern and phenotypic traits

Potential correlations between phenotypical traits and the recurrence profile of the strains were investigated in this chapter. We have observed that approximately 60% of the recurrent and sporadic strains could not survive to a concentration beyond 0.25 mM PQ or H_2O_2. No significant difference was found between recurrent vs. sporadic strains in their oxidative stress survival responses ($p > 0.05$, Figure 37) nor in their acclimation to aerobic conditions capacities ($p > 0.05$). Nonetheless, a correlation between the recurrent profiles and the adhesion/biofilm ability was observed: the recurrent strains have a significant higher adhesion/biofilm-forming capacity than the sporadic ones (chi-squarest test, $p = 0.0266$) (Figure 38).

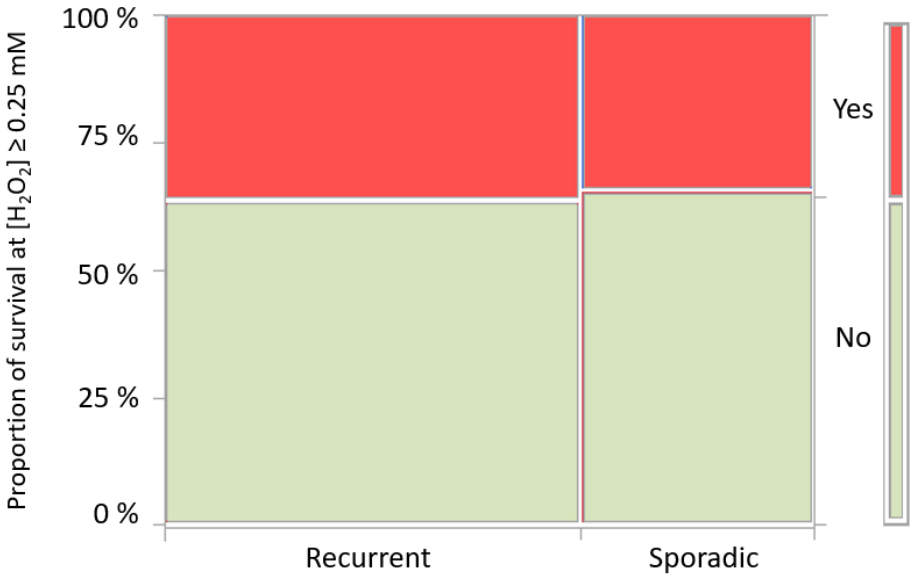


Figure 37: Absence of correlation between survival H_2O_2 (≥ 0.25 mM) and the recurrence profile of the strains.
The same trend was observed for PQ.

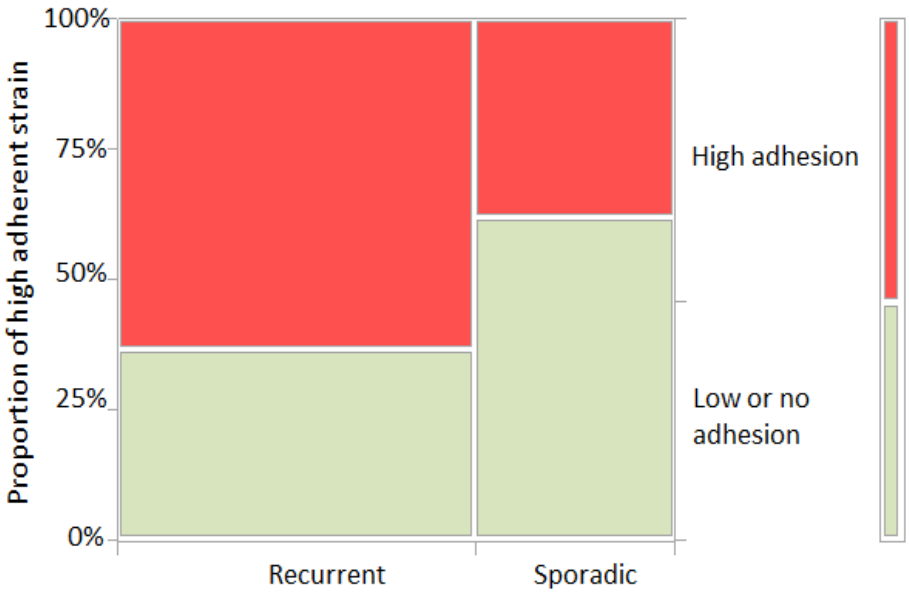
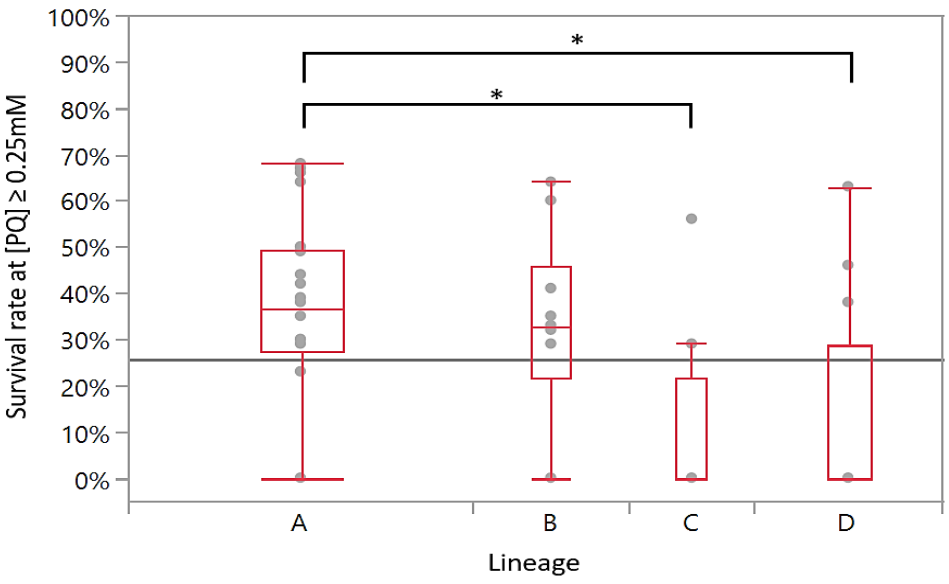


Figure 38: Correlation between adhesion capability and the recurrence of the strains.
The same trend was observed for biofilm formation.

2.3. Correlation between survival rate to oxidative stresses and genomic lineages

As abovementioned, the susceptibility to oxidative stresses do not contribute to the variations observed between the recurrent vs sporadic strains. As lineages are significantly genetically distant one from another, correlations between the different genomic lineages and oxidative stress profiles were explored. Interestingly, the statistical analyses indicate that lineage A is significantly more resistant to PQ stress than lineages C and D (ANOVA, p = 0.0393 and p = 0.0234, respectively) (Figure 39 A). Strains belonging to lineage A survived significantly more to 0.25 mM H_2O_2 than strains belonging to lineage D (ANOVA, p = 0.0151) (Figure 39 B). Strains of the lineage B demonstrated a resistance to a concentration beyond 0.25 mM H_2O_2 significantly higher than strains from lineages C and D (ANOVA, p = 0.0171 and p = 0.0015, respectively) (Figure 39 B).

A



B

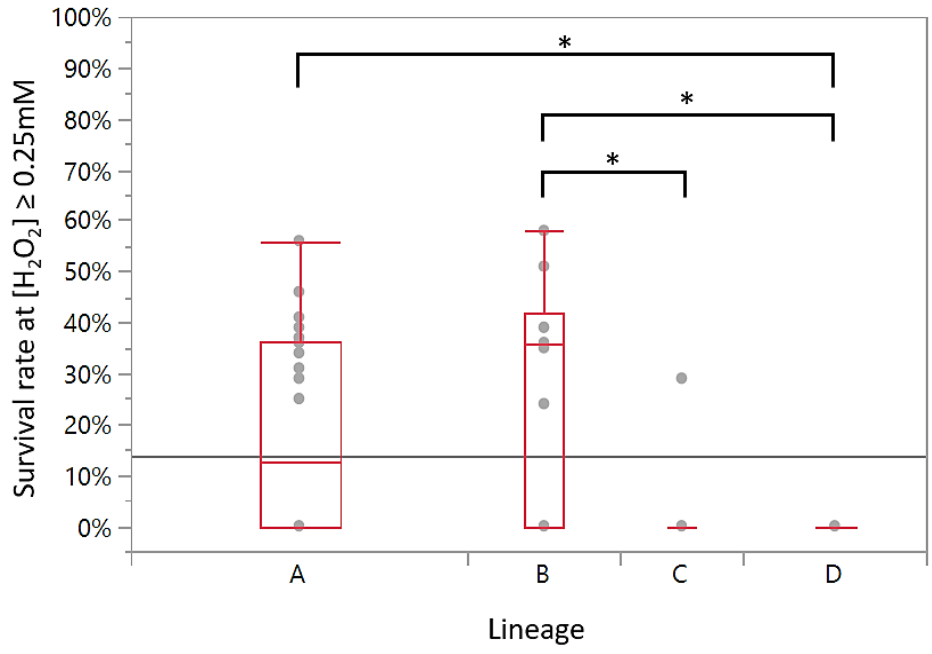


Figure 39: Distribution of the survival rate of the 52 recurrent *C. jejuni* strains exposed to PQ (A) and to H_2O_2 (B) according to their genomic lineage.
Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

2.4. Correlation between adhesion and biofilm formation capacities according to genomic lineages

As previously revealed, recurrent strains had a significant higher adhesion/biofilm-forming capacity than sporadic strains. Correlation between adhesion and biofilm formation abilities were therefore explored deeper according to the genomic lineages and the different categories of adhesion and biofilm formation determined in chapter III. According to the significant *p*-values obtained between each lineage, it appeared that each lineage had an adhesion significantly different from the three other lineages (Figure 40, Table 14). Overall, lineages C and D had an adhesion significantly higher than the one for lineages A and B (ANOVA, *p*-values in Table 14).

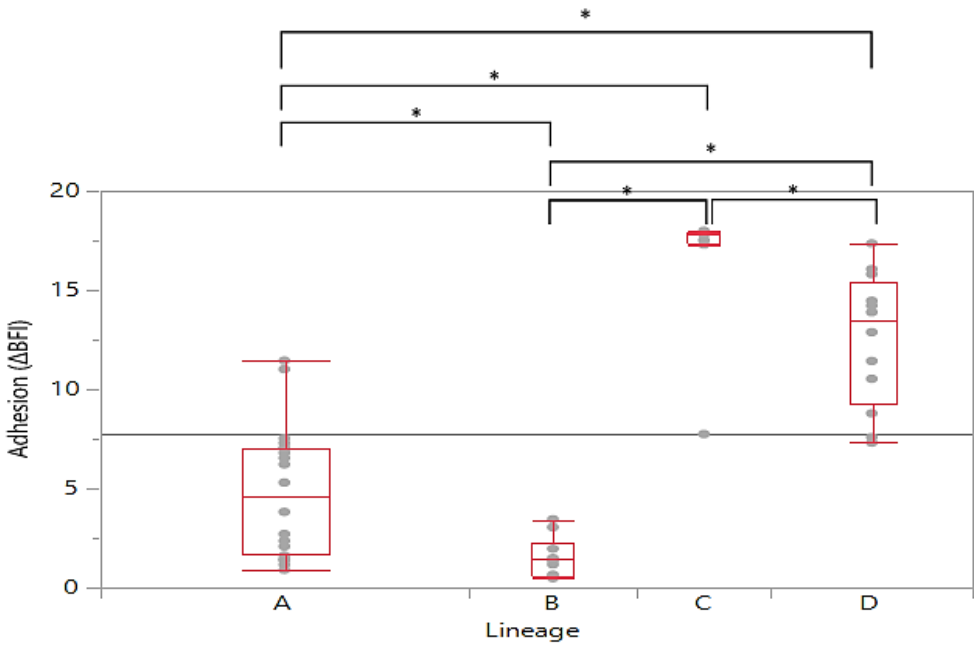


Figure 40: Distribution of adherent strains among the 52 recurrent *C. jejuni* strains according to their genomic lineage.
Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

Table 14: Comparison of the adhesion capacity between the four recurrent lineages tested.

lineage vs. lineage		*p*-value (ANOVA test)
A	D	< 0.0001
A	C	< 0.0001
A	B	0.0375
B	D	< 0.0001
B	C	< 0.0001
C	D	0.0290

Concerning the distribution of biofilm producers among the strains according to their genomic lineages, it appeared that lineages A and B had a significant lower capability to develop biofilm (Figure 41). Surprisingly, lineage C was not classified as a high biofilm producer (ΔBFI median value around 2) although it displayed a significant higher adhesion ability than lineages A and B (Figure 40, Table 14, and Figure 41). In contrast, lineage D was classified as a high biofilm former with a biofilm-forming capability significantly higher than the one for lineages A, B, and C (ANOVA, $p < 0.0001$ for all three). There was also a significant difference in biofilm formation between lineages B and C, with lineage B being less biofilm-producer than lineage C (Figure 41).

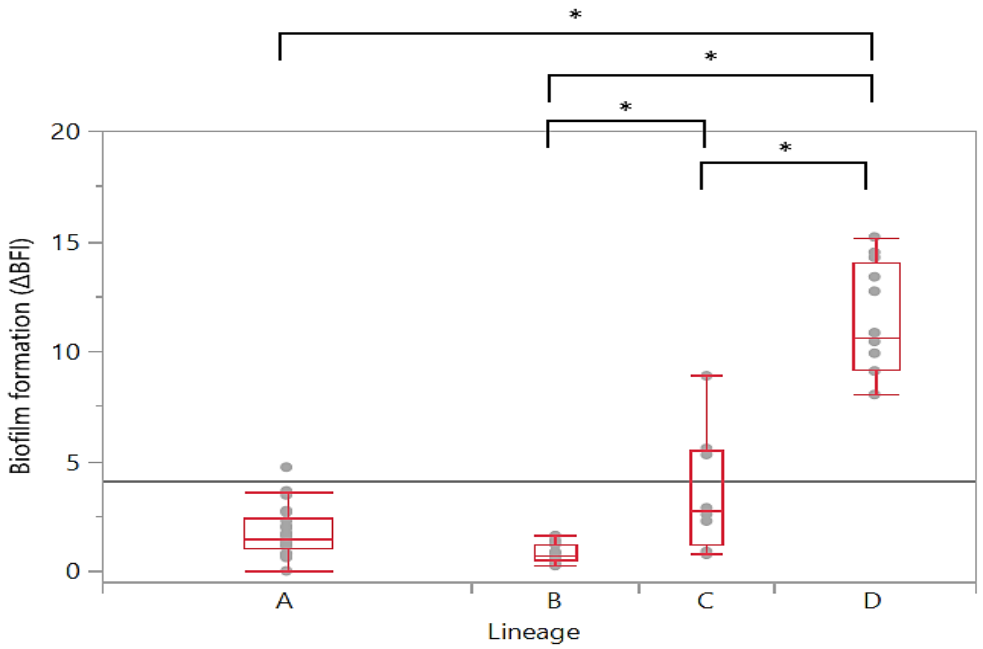


Figure 41: Distribution of biofilm producers among the 52 recurrent *C. jejuni* strains according to their genomic lineage.
Statistical significance was determined using the ANOVA test (*$p \leq 0.05$).

2.5. Correlation between acclimation to aerobic conditions and genomic lineages

The sampling design has insufficient power to validate the statistical model for acclimation to AC because of a lack of representation in each genomic lineage. Consequently, no statistical correlation could have been performed between acclimation ability and genomic lineages identified. Nonetheless, a correlation between acclimation to AC and the belonging to the different recurrent lineages (chi-squared test, p =

0.0135) was observed, which indicates the existence of at least one significant difference between the lineages. It goes towards a trend for acclimation to aerobiosis for lineages A and D. To strengthen this result and increase the reliability, the number of strains in some groups should be increased.

2.6. Distribution of the fluoroquinolones resistance according to the genomic lineages

A high proportion of strains belonging to the lineages A (CC ST-21), B (CC ST-257), C (CC ST-464), and D (CC ST-21) was predicted resistant to FQ (Figure 42). As expected, no correlation between the resistance to FQ and the genomic lineages was established (chi-squared test, $p > 0.05$). Therefore, fluoroquinolone resistance prediction could not discriminate different phenotypical behaviours among the genomic lineages.

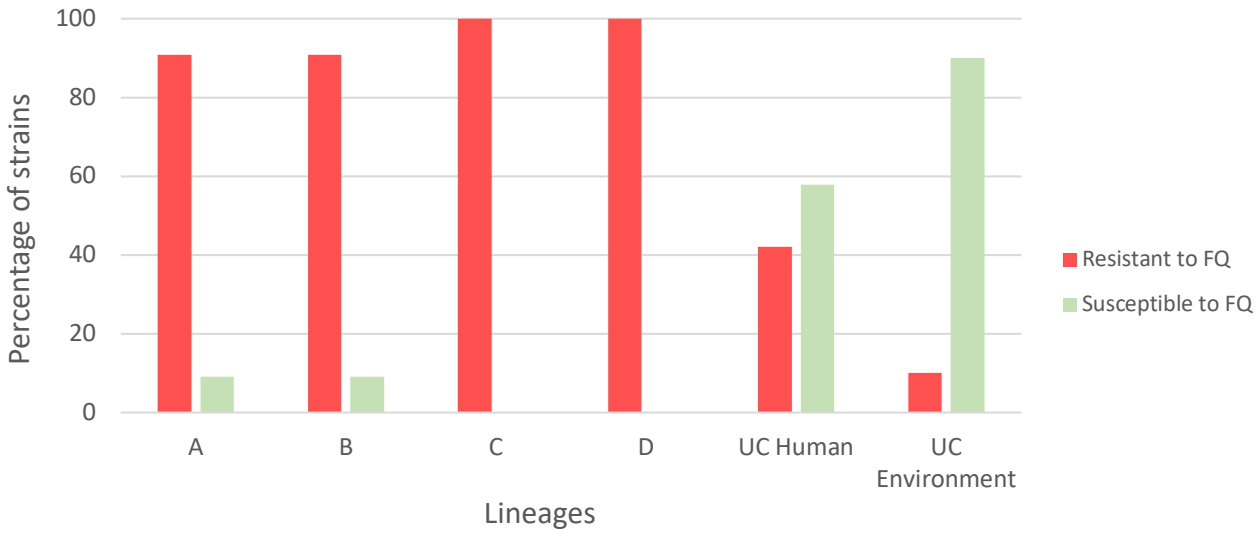


Figure 42: Distribution of the FQ resistance prediction according to the genomic lineages.

2.7. Correlation between targets of Venn diagram, phenotypes, and epidemic profiles of each genomic lineages

According to our results, all the characterized phenotypical behaviors related to persistence and transmission (oxidative stress responses, acclimation to aerobic conditions, and biofilm development) were independent. However, correlation analyses indicate that specific behavior, including independent

phenotypes, could be attributed to each recurrent lineage resulting in a metaphenotypical profile for each lineage (Figure 43). The metaphenotype of lineage A is composed of the survival to oxidative stress and no or low adhesion and biofilm formation, with a tendency for the acclimation to AC. Concerning the lineage B, its metaphenotype is reflected by the survival to oxidative stress, without the ability to adhere, nor to form a biofilm, and a tendency to acclimate to AC. The metaphenotype of lineage C included the susceptibility to oxidative stress and a high adhesion capacity with no biofilm formation ability and a tendency to not acclimate to AC. Lineage D metaphenotype consisted of the susceptibility to oxidative stress, a high adhesion and biofilm formation capacities, and a tendency for the acclimation to AC. Within the CC ST-21, two different metaphenotypes are distinguished: the one linked to the lineage A (survival to oxidative stress, no adhesion, no biofilm formation) and the one linked to the lineage D (susceptibility to oxidative stress, high adhesion and biofilm formation capacities) (Figure 43).

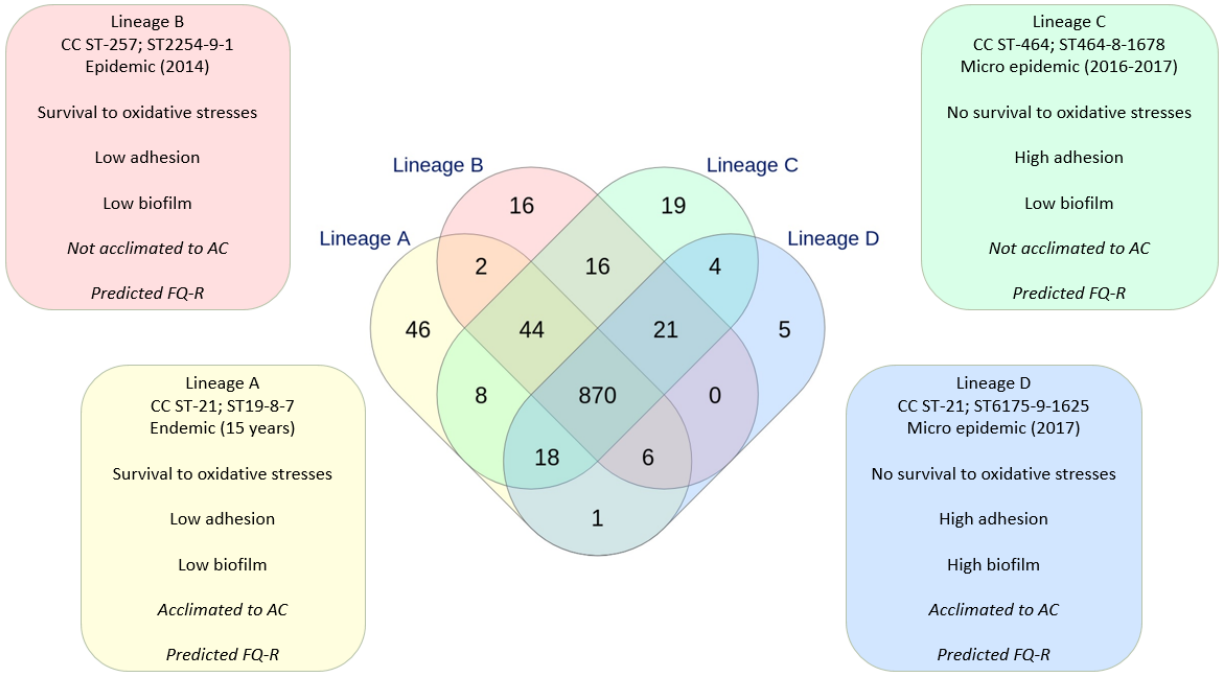


Figure 43: Metaphenotypical profile for each recurrent genomic lineage and the number of specific targets related to them.

Specific targets were obtained from Venn diagram performed with wgMLST INNUENDO typing scheme (see in chapter II: Gene-by-gene WGS analysis). Phenotypes about AAC are mentioned in italic, as they were not validated by the statistical model due to insufficient distribution of the data. Phenotypes about fluoroquinolone resistance (FQ-R) are mentioned in italic, as they are only *in silico* predictions.

2.8. Identification of the unique targets for each the genomic lineage

No difference for phenotype characterization was observed between lineages A and B if we discard results concerning acclimation to aerobiosis as it has to be reinforced with additional experiments, as the statistical model could not be fully validated. As two different targets were identified between lineages A and B (Table 15), this indicates that the open reading frames (ORF) included in these two targets (Cj1089c and Cj0243c) could contribute to the oxidative stress survival, to the absence of a high adhesion or a high biofilm formation. These two targets include conserved proteins of unknown function, i.e., proteins identified in a large number of strains without known function. Further investigations are required on these proteins to elucidate their function in *C. jejuni*.

Table 15: Shared targets between lineages A and B (n = 2) identified with the wgMLST INNUENDO typing scheme, and corresponding ORF according to MAGE, KEGG, and UniProt data.

Shared targets	MAGE / KEGG / UniProt identification
group_4957 / Cj1089c	Hypothetical protein / conserved protein of unknown function
group_8313 / Cj0243c	Hypothetical protein / conserved protein of unknown function

Concerning the oxidative stress response, a significant resistant trend was identified for lineages A and B while a significant susceptible trend was observed for lineages C and D. If the oxidative stress susceptibility phenotype results from the same cellular mechanism regardless of the lineages, then targets shared by lineages A and B and not by lineages C and D would help to explain the oxidative stress resistance phenotype and targets shared by lineages C and D would contribute to explain the oxidative stress sensitivity phenotype. Only two targets were identified to be common to lineages A and B (Table 15) and not to lineages C and D, and four targets were identified to be common to lineages C and D and not to lineages A and B (Table 16). In the latter targets, the *rfbE* gene encodes for RfbE protein, a sugar-nucleotide epimerase-dehydratase catalyzing the following reaction: cytidine diphosphate-3,6-dideoxy-D-glucose → cytidine diphosphate-3,6-dideoxy-D-mannose, and the TssB and TssC proteins compose the contractile sheath of the T6SS. Interestingly, two targets including genes involved in the T6SS are shared by strains of lineages C and D.

Table 16: Shared targets between lineages C and D (n = 4) identified with the wgMLST INNUENDO typing scheme, and corresponding ORF according to MAGE, KEGG, and UniProt data.

Shared targets	MAGE / KEGG / UniProt identification
rfbE	Amino sugar and nucleotide sugar metabolism
group_11845	TssB - Type VI secretion system contractile sheath large subunit
group_13120	Conserved protein of unknown function
group_9775	TssC - Type VI secretion system contractile sheath large subunit

Phenotype characterization indicates a difference in the ability to form a biofilm: strains of lineage D are able to form biofilms while those of lineage C are not, although they both display a high capacity for adhesion. Among the targets they shared, two were related to the T6SS. They could explain their adhesion capacity as they compose the contractile sheath of the T6SS (Table 16). Furthermore, in lineage C only, the *mshL* gene encodes for the pilus biogenesis protein MshL. Pili have been shown to play a key role in cell-cell, or cell-abiotic surfaces interactions in bacteria (Piepenbrink and Sundberg, 2016), thus this protein could be involved, in a similar manner, in the enhanced adhesion determined in *C. jejuni* strains belonging to lineage C. Furthermore, 19 unique targets were identified for lineage C (Table 17) and 5 for lineage D (Table 18) that could potentially explain the difference in the biofilm development. In lineage C, within these 19 specific targets, a repressor, i.e., a DNA-binding protein that inhibits the expression of one or more genes by blocking the attachment of the RNA polymerase to the promoter, was identified. It could be involved in the biofilm formation repression as demonstrated previously for the CprS sensor kinase, which downregulates the biofilm growth (Svensson et al., 2009). In this lineage C, there were also three other targets related to genes encoding for T6SS elements (*tssG*, *hcpA*, and the ImpG/VasA protein). Based on a nucleotide-level analysis, these targets were detected to be specific to lineage C. After blastP analyses based on data extracted from the NCBI RefSeq genome database, these proteins were also found in lineage D. The difference was detected in a lower homology that distinguished them from lineage C strains with 64%, 62%, and 89% of protein identity. This difference could concern a specific part of the proteins or their 3D-conformation. Concerning the lineage D, two of the five unique targets correspond to enzymes: RloA and serine dehydratase. RloA is an ATPase, while the serine dehydratase is involved in the formation of glucose from non-carbohydrate precursors, such as pyruvate, amino acids and glycerol.

Table 17: Unique targets identified in lineage C (n = 19) with the wgMLST INNUENDO typing scheme, and corresponding ORF according to MAGE, KEGG, and UniProt data.

Unique targets	MAGE / KEGG / UniProt identification
kfiD	Amino sugar and nucleotide sugar metabolism
mshL	Pilus (MSHA type) biogenesis protein MshL
yhdJ	Methyltransferase
group_10001	FHA domain-containing protein
group_11835	Conserved membrane protein of unknown function
group_13966	Conserved protein of unknown function
group_14704	Amino sugar and nucleotide sugar metabolism
group_15078	Conserved protein of unknown function
group_15677	Pentapeptide repeat-containing protein
group_29823	Conserved protein of unknown function
group_35838	*tssG* gene – Type VI secretion system
group_36080	Protein ImpG/VasA – Type VI secretion system
group_36088	*hcpA* – Type VI secretion system effector
group_4798	Conserved protein of unknown function
group_5195	Repressor
group_5403	Type III restriction-modification system methylation subunit
group_5662	Conserved protein of unknown function
group_7883	*hddC* gene – guanosyltransferase
group_8100	Conserved protein of unknown function

Table 18: Unique targets identified in lineage D (n = 5) with the wgMLST INNUENDO typing scheme, and corresponding ORF according to MAGE, KEGG, and UniProt data.

Unique targets	MAGE / KEGG / UniProt identification
group_12450	*rloA* - AAA family ATPase
group_13193	Conserved protein of unknown function
group_7310	Conserved protein of unknown function
group_8186	*sdaC* - serine:H(+) symporter SdaC / serine dehydratase
group_8590	*cpp13*

As the lineage A displayed an endemic pattern, it could be of interest to analyze its 46 unique targets identified. Its specific targets encode for enzymes such as a DNA primase (encoded by *dnaG*), a peptidase, or different methyltransferases. Moreover, five proteins are involved in diverse biosynthesis pathways, and eight others are originated from phages (e.g., a phage integrase encoded by *intA*). In total, 19 proteins are conserved which function that has not been yet elucidated (Table 19).

Table 19: Unique targets identified in lineage A (n = 46) with the wgMLST INNUENDO typing scheme, and corresponding ORF according to MAGE, KEGG, and UniProt data.

Unique targets	MAGE / KEGG / UniProt identification
dnaG.	DNA primase
hsdS	Type I restriction-modification system, specificity subunit S
intA	Phage integrase family site specific recombinase
*kdsB*_2	Nucleotidyltransferase
group_10703	Phage gp6-like head-tail connector protein
group_12436	Peptidase
group_13054	*trkA* domain protein
group_13131	Putative DNA repair protein rad2
group_13906	Putative DNA binding protein
group_13909	Transcriptional regulator
group_13920	Type II toxin-antitoxin system PemK/MazF family toxin
group_14145	HNH endonuclease domain protein
group_14299	Pyrophosphorylase
group_15204	Putative phage head-tail adaptor
group_3144	Amino sugar and nucleotide sugar metabolism
group_36028	Putative phage head-tail adaptor
group_36493	*tagF* - glycerophosphotransferase
group_36503	Putative capsular polysaccharide biosynthesis protein
group_36589	Transmembrane protein
group_38059	*tarD* - Glycerol-3-phosphate cytidylyltransferase
group_4355	Adenine-specific methyltransferase (*hsdM*)
group_5331	ATP/GTP binding protein
group_5341	DNA-binding protein
group_8323	*hsdM* - methyltransferase
group_8653	Putative enzyme
group_8666	Major capsid protein, HK97 family
group_9382	Putative phage terminase, small subunit
group_9785	Phage protein, HK97 gp10 family
Conserved proteins of unknown function: group_6032, group_8181, group_8356, group_8382, group_8653, group_10186, group_10187, group_10694, group_11268, group_11277, group_11853, group_12397, group_12440, group_13128, group_13136, group_13914, group_15203, group_37855, group_38362	

2.9. Identification of the T6SS core components

A fully assembled T6SS is composed of a minimal of 13 core components: *tssJ*, *tssK*, *tssL*, *tssM*, *tssA*, *tssB*, *tssC*, *hcp*, *tssE*, *tssF*, *tssG*, *clpV*, and *vgrG*, 12 being identified in few *C. jejuni* strains and one being absent (*cplV*) (Figure 44) (Robinson et al., 2021).

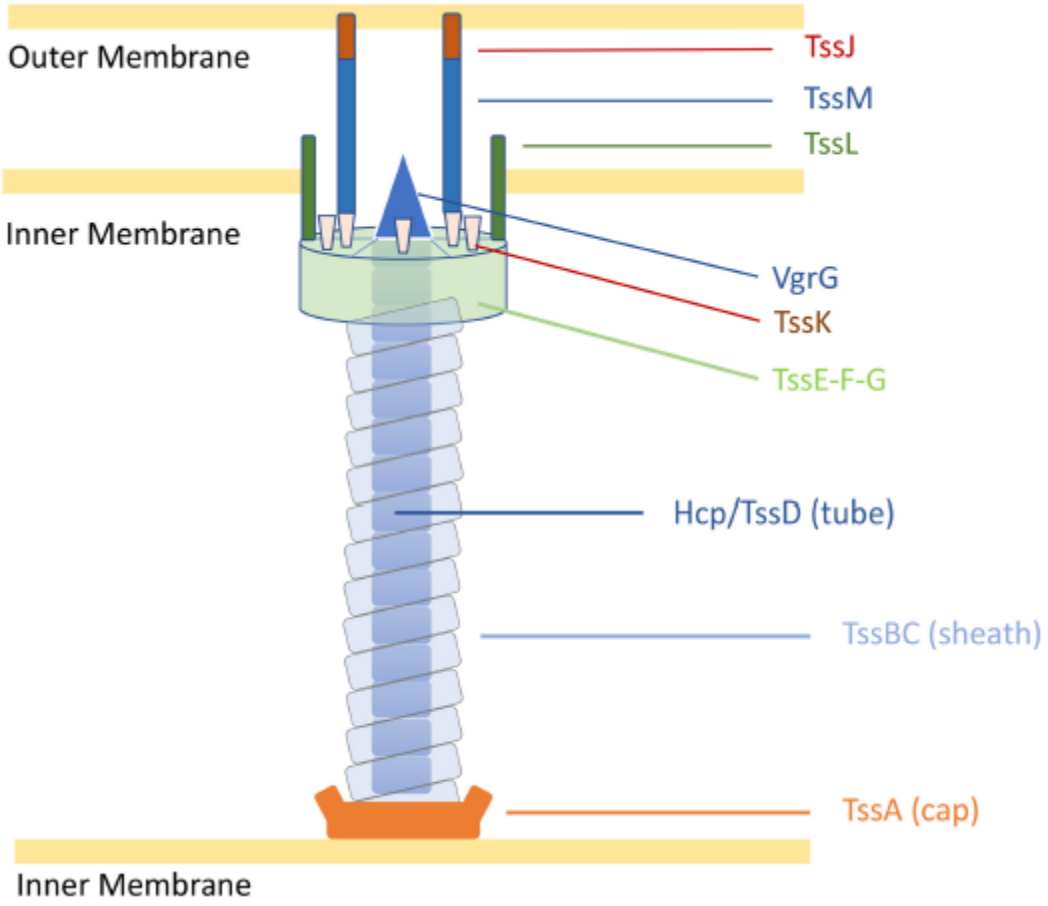


Figure 44: Representation of the *C. jejuni* T6SS core components (extracted from **(Robinson et al., 2021)**).

Further analyses of the sequencing data highlighted that 12 out of 13 core components were present in lineages C and D only. Their identification resulted in 89% to 100% identity using the UNIPROT reference through the blastP analyses. For instance, 100% identity for HcpA of the strains belonging to lineages C and D was observed (Figure 45 and Figure 46). The missing subunit in these strains as compared to T6SS in general was the ATPase named ClpV or TssH, as previously shown in *C. jejuni* (Robinson et al., 2021). In parallel, none of the T6SS core components was found in lineages A and B.

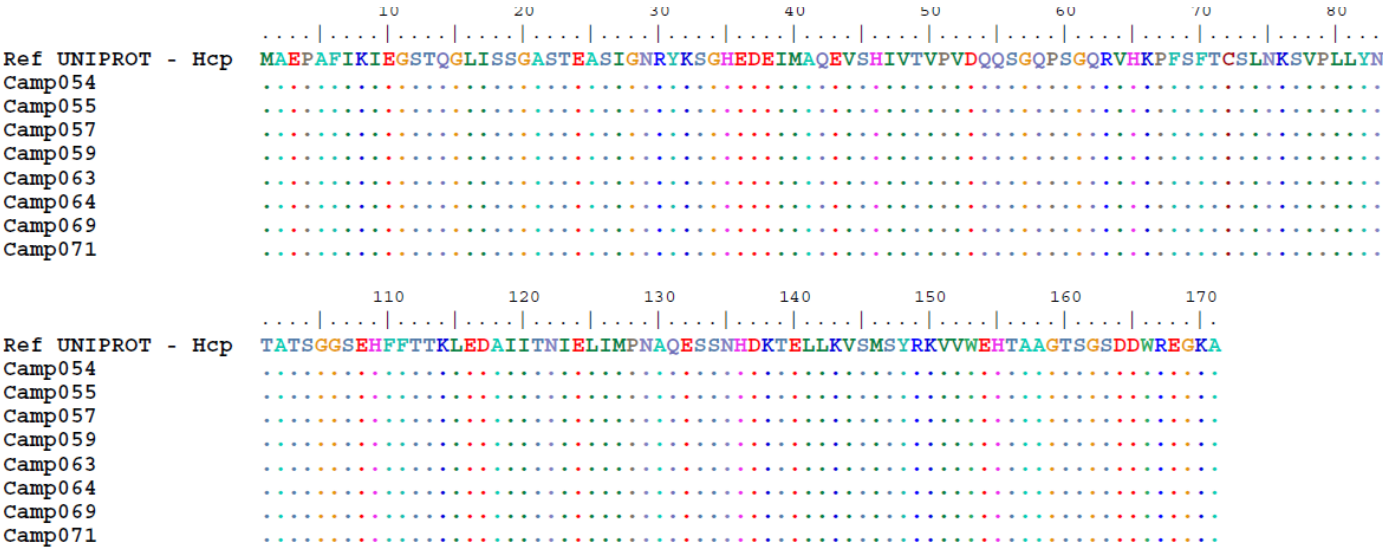


Figure 45: Protein sequence alignments for Hcp in strains belonging to lineage C.

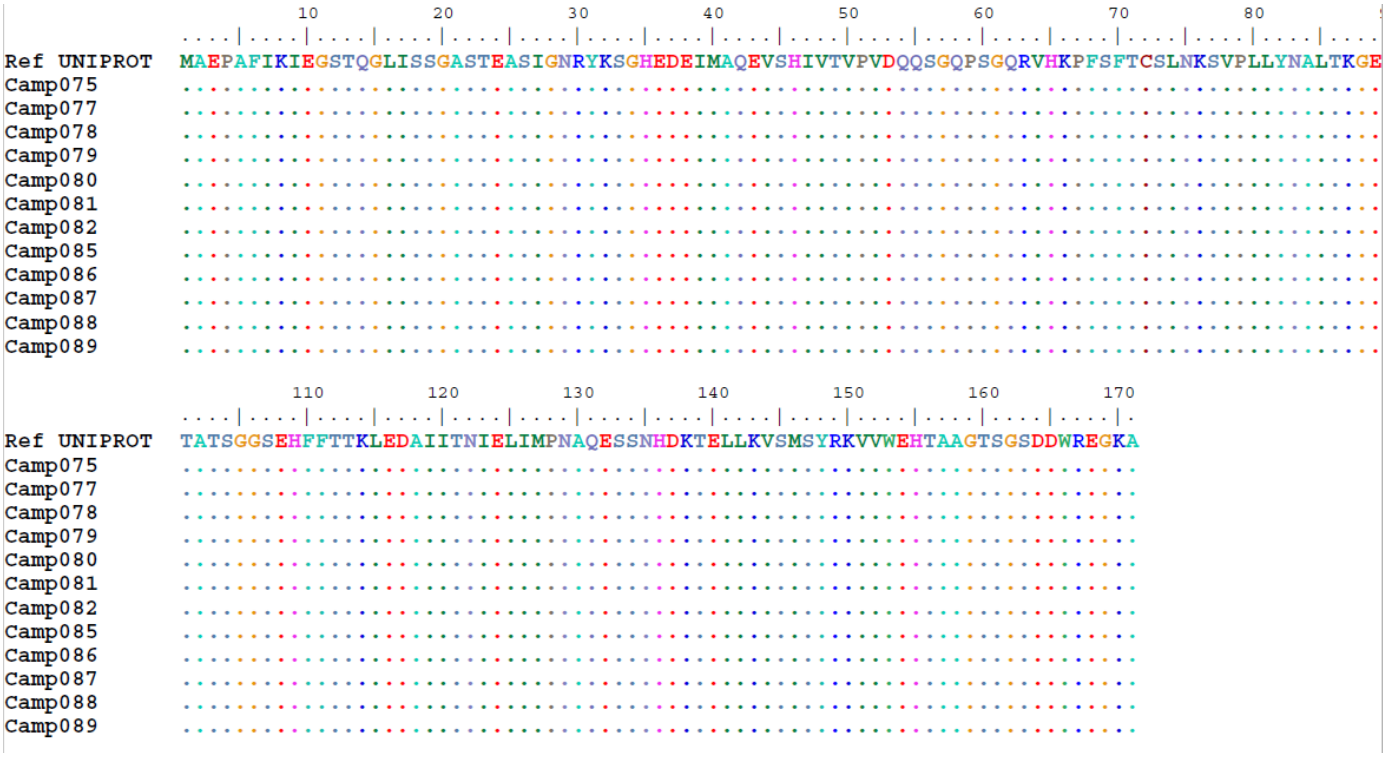


Figure 46: Protein sequence alignments for Hcp in strains belonging to lineage D.

3. Discussion

In chapter II, we pinpointed the presence of monomorphic lineages regularly infecting humans with a broad distribution over time and sources, suggesting persistence in reservoirs as well as in the environment. It points out the possible long-term existence of stable clones appearing through the concordance of the different typing schemes. As the result to adaptive response, in chapter III, the screening of *C. jejuni* strains showed that these strains could be classified according to their ability to respond to diverse harmful conditions related to aerobiosis coping. This work aimed at investigating whether particular phenotypical traits could be related to these recurrent genotypes. The selected *in vitro* assays investigated the *Campylobacter*'s ability to tolerate oxidative stress, to multiply in aerobic conditions, to adhere to an inert surface, and to develop a biofilm. In addition, resistance to quinolone inferred from sequence data is discussed as information completing the phenotypic profile of the strains.

The phenotypic tests were performed on a strain collection, including those related to the four genomic lineages recurrently identified in human infection in Luxembourg, as well as on an outgroup panel corresponding to sporadic strains. Lineages A and B are the most represented in our national surveillance database and are linked to an endemic profile occurring over 15 years in human infections and the most large-national scale epidemic curve ever identified, respectively. In addition, lineages C and D, displaying micro-epidemic and emerging patterns, were also identified using genomic-scale comparisons in chapter II. Lineages A and D belong to Clonal Complex ST-21 (CC ST-21), defined as "host generalist", while lineages B and C, belonging to ST-257 and ST-464 CCs, are more adapted to the chicken reservoir and described as "host specialist" (Sheppard et al., 2014). In a first step, results were explored by comparing the response variations according to the patterns "recurring" and "sporadic". The survival to oxidative stress did not appear to discriminate the long-term occurrence of the strains, and the analysis of the correlation between the acclimation to aerobic conditions and the recurrence pattern was inconclusive, due to the invalidation of the statistical model. Interestingly, adhesion to inert surface and biofilm development were only correlated to recurrent strains.

Consequently, phenotypic responses were analyzed according to genomic lineages; correlations between genetic profiles and physiological capacities were examined. Lineages A and B are characterized by a marked survival ability to oxidative stress but they are not able to adhere and cannot form biofilms. In contrast, lineages C and D did not significantly survive oxidative stresses. In addition, more adherent-proficient genotypes with the proven ability to form biofilm was observed only for lineage D. Concerning the survival to pro-oxidant stresses, the results for lineages A and C are consistent with the results of

Mouftah et *al.* (2021), who found that host generalist strains have a greater tolerance to peracetic acid, which can be assimilated to H_2O_2 stress as this molecule is formed upon peracetic acid degradation (Mouftah et al., 2021). Moreover, Pascoe *et al.* (2015) found that a higher proportion of host generalist isolates exhibited biofilm formation than broiler specialist isolates, which is in accordance with the results of lineages B and D strains (Pascoe et al., 2015) but not with the host generalist lineage A. As no correlation could be found between the different phenotype tested, the absence of interdependency could not segregate the genomic lineage according to only one phenotype. However, this study revealed that a genomic lineage could be characterized by a specific combination of independent phenotypes. In consequence, we suggest that each genomic lineage is described by a metaphenotype representing a specific combination of independent phenotypes.

The analyses of the different metaphenotypes was performed according to the specific targets revealed by the Venn-diagram approach to investigate those that could be involved in. Within the specific set of targets identified for lineage A, eight belonged to phage origin including phage integrase, phage protein, phage gp6-like head-tail connector protein. The bacteria-phage coevolution is known as a driver of ecological and evolutionary processes in microbial communities (Koskella and Brockhurst, 2014). Some *C. jejuni* isolates harbored CJIE1 prophage, whose presence was strongly linked to specific phenotypic behaviors (e.g., invasion abilities) (Clark et al., 2016). Another target identified in lineage A belongs to type II toxin-antitoxin (TA) system: the type II TA system PemIK/MazEF family toxin. MazEF is a TA module widely distributed among many bacterial species, such as *Escherichia coli, Staphylococcus* genus, or *Campylobacter* spp. (Yan et al., 2012; Bukowski et al., 2017). TA modules consist of a pair of antagonistic genes that encode for a stable toxin and an unstable antitoxin. Recent studies have demonstrated that they play a central role in bacterial persistence (Page and Peti, 2016). Thus, these genetic determinants could play a role in the long-term occurrence of the lineage A, which is defined as endemic for at least 15 years in Luxembourg.

The T6SS is a secretion system allowing the delivery of protein effectors to diverse prokaryotic cells and thus, participating in inter-bacterial competition and pathogenesis (Zoued et al., 2014). Studies examining the prevalence of the T6SS in *C. jejuni* were performed in Europe and a large variation in prevalence was observed: in 2014, approximately 3% of chicken isolates from the United Kingdom were T6SS-positive, whilst 28.8% of similar isolates were T6SS-positive in Northern Ireland in 2015 (Harrison et al., 2014; Corcionivoschi et al., 2015). Kovanen *et al.* (2019) analyzed the T6SS presence in wild birds, and half of western jackdaw isolates and more than 70% of mallard duck isolates were T6SS-positive in 2018

(Kovanen et al., 2019), suggesting that the T6SS is becoming increasingly prevalent in *C. jejuni* strains. The presence of 12 out of 13 core components of the T6SS was highlighted in the metaphenotypes of lineages C and D. The core component TssH, also named ClpV, was not found in any *C. jejuni* strain as previously described in *C. jejuni* harboring the T6SS (Liaw et al., 2019; Robinson et al., 2021). This missing protein in *C. jejuni* is an ATPase responsible for the disassembly of the contracted sheath components. Despite its absence, the presence of a functional T6SS was demonstrated in other organisms (e.g., *Helicobacter hepaticus* or *Salmonella* Typhimurium) as well as in *C. jejuni* (Lertpiriyapong et al., 2012; Sana et al., 2016; Kanwal et al., 2019). According to Liaw *et al.* (2019), this raises the possibility of an alternative mechanism for the sheath disassembly, or the existence of a ClpV-like ATPase encoded near from the T6SS cluster (Liaw et al., 2019). Interestingly, one of the unique targets identified in lineage D was an ATPase called RloA. This enzyme was detected near the *vgrG* gene, encoding one of the core components. The RloA protein is a member of the AAA+ superfamily, which is composed of proteins involved in a variety of different functions, such as protein unfolding and degradation (Snider et al., 2008). The protein was also mentioned by Robinson et *al.* (2021) as a serious potential alternative to ClpV (Robinson et al., 2021). This possibility suggests the presence of a complete and functional T6SS in metaphenotype of lineage D but not in the other metaphenotypes. In *C. jejuni*, the T6SS biological functions have been associated with host colonization, cell adhesion and invasion, and contributing to oxidative stress defense (Lertpiriyapong et al., 2012; Liaw et al., 2019). It is a protein transport machinery involved in niche establishment (i.e., it may provide bacteria niche-specific adaptive capacities). Indeed, its secreted effectors display a wide range of functions (e.g., nucleases, lipases, or pore-forming activities), and their injection into competing bacteria promotes the fitness of T6SS-positive strains in polymicrobial environments (e.g., the gut ecosystem), facilitating their survival (Jiang et al., 2016; Coulthurst, 2019; Jana et al., 2019; Fridman et al., 2020; Wood et al., 2020). Furthermore, it has been demonstrated that the T6SS has a diverse impact on biofilm formation in various species: it could enhance it (e.g., in *Acinetobacter baumannii*) or be implicated in the maturation phase (e.g., in *Pseudomonas fluorescens*) (Gallique et al., 2017; Kim et al., 2017). Regarding the latter, the NCTC 11168 strain was shown to be deficient in entering the biofilm maturation phase, which could explain why some strains can adhere and cannot form a biofilm (Turonova et al., 2015). Within the T6SS components, Noreen *et al.* (2018) also highlighted one of the roles of the hemolysin-corregulated protein (Hcp), also called TssD, in *C. jejuni*: it could act as an autoinducer for biofilm formation (Noreen et al., 2018). As metaphenotype of lineage D is characterized by the capability to form biofilm in contrast to metaphenotype of lineage C, then the presence of T6SS with RloA to complete its

functionality might be involved in biofilm growth and maturation in *C. jejuni.* It could therefore be a potential molecular marker to distinguish strains able to develop a biofilm among *C. jejuni* population.

As previously mentioned, strains belonging to lineage D are characterized by a high adhesion ability and biofilm formation. They also have a tendency to better acclimate to aerobic conditions. Within the unique targets identified for this lineage, two are of interest: *sdaC* and *cpp13*. The *sdaC* gene is organized in an operon with *sdaA*, and an active SdaA protein was demonstrated as essential for colonization of the avian gut by *C. jejuni* (Velayudhan et al., 2004). Furthermore, the *cpp13* gene was found in the pTet plasmid in the strain 81-176 and it appeared that it could contribute to *C. jejuni* fitness (de Vries et al., 2017). Lineage D has emerged from our surveillance activities since 2017. According to its particular phenotypical capacities and its genetic content (i.e., the tendency to acclimate to aerobic conditions, the high adhesion/biofilm formation abilities and the presence of a potential complete and functional T6SS), the spread of this lineage should be further investigated to determine whether it is a clonal expansion that is sustained over time.

Considering AMR prediction, a high percentage of the strains were predicted resistant to FQ within the selected panel. AMR pattern of *C. jejuni* influences its survival by providing an improved biological fitness, for instance; e.g., multi-drug resistant *C. jejuni* strains were among the longest surviving isolates in Good *et al.* work (2019) (Luo et al., 2005; González and Hänninen, 2012; Good et al., 2019). Furthermore, the ability of FQ-resistant strains to persist in the environment (e.g., in poultry production), after the removal of the selective pressure, was already demonstrated (Price et al., 2007b). With the increase of AMRs among isolates from clinical, veterinary, and environmental sources, *C. jejuni* was classified as a serious antimicrobial resistant threat. Many studies were led to define the frequency of resistance among specific phylogenetic lineages. Indeed, even though AMRs were distributed among isolates belonging to relative distant lineages, indicating a widespread dispersal; Wimalarathna and colleagues also brought evidence of clustering of resistance phenotypes within lineages, pointing out local expansion of resistant strains (Wimalarathna et al., 2013). A study performed in Slovenia determined antibiotic resistance and MLST profiles of 52 *C. jejuni* isolates from diverse sources (e.g., human, animal, chicken meat, and water), showing the high incidence of FQ-resistant strains within CC ST-21 rather due to clonal spreading than high genetic plasticity (Kovač et al., 2014). This high prevalence in drug-resistance within CC ST-21 is in accordance with our results, as 32 out of 34 strains belonging to lineages A and D, belong to CC ST-21 and are FQ-resistant. Association study between ST and quinolone resistance was also performed on strains belonging to CC ST-464 (lineage C in our study) (Cody et al., 2012). The increase of

their relative incidence in Europe, as well as their association with ciprofloxacin resistance, was also confirmed later by Kittl and colleagues (Kittl et al., 2013). The same phenomenon was observed in our results, as all the strains belonging to CC ST-464 are FQ-resistant. Furthermore, this lineage was reported in a study performed in USA: patients reporting travel history were the more likely risk exposure linked to infections caused by this ST (Cha et al., 2016). These results could indicate the persistence of this FQ-resistant lineage and its dissemination outside of Europe among years.

According to our analyses, combination of independent biological responses was found specifics to each lineage. These data suggest that a combination of different phenotypical abilities, so called here the metaphenotype, may contribute to the adaptation and survival of these stable clones over time and from various sources. These results also tend to indicate the existence of a selective pressure behind the emergence and persistence of the most adapted and resistant lineages to different environmental stresses. Survival strategy of bacterial pathogens outside the host could lead to the selection of specific genetic profiles and changes in gene expression. Reversible switches in the expression of genes (i.e., phase variation) have been reported in *C. jejuni*, leading to numerous phenotypes (Bayliss et al., 2012). Within the unique targets identified in lineages A, C, and D, specific methyltransferases were found in each of them. These enzymes are involved in DNA methylation, which regulates the reversible switching (phase variation) of gene expression in many bacterial species, a mechanism that produces phenotypic variations. The emergence of epigenetic lineages allows the adaptation of bacterial communities to challenging environments and to moderating the behavior of pathogenic agents (Sánchez-Romero and Casadesús, 2020). For instance, DNA methylation could be an avenue to consider the acclimation to AC trend of *C. jejuni* strains. On the other hand, the emergence of clones can hardly be explained by a single biological advantage, as this phenomenon is rather multifactorial. Indeed, the different phenotypic responses did not exhibit interdependence, and lineages were rather characterized by a metaphenotype. This work highlighted some potential molecular markers and gave some avenues to understand better the behavior of *C. jejuni* and to control the spreading of these clones, which are responsible for half of the human infections in Luxembourg. Further analyses should concentrate efforts on understanding the molecular mechanisms underlying these metaphenotypes in *C. jejuni,* including the regulation of several genes involved in the different phenotypical responses. The survival and persistence of *C. jejuni* in the environment enhance effectiveness of its transmission to human in term of risk exposure and thus represent a public health risk. Further experimentations and analyses might focus on metaphenotypes related to lineages exhibiting a clonal expansion and associated with human infections.

Chapter V: General discussion, conclusion, scientific outlooks and economic outcomes

1. Reminder of the objectives and contextualization of the project

Campylobacteriosis ranks third in cause of death in absolute terms behind listeriosis and salmonellosis, due to its high notification rate in Europe (64.1 cases per 100,000 population) as well as in Luxembourg (103.8 cases per 100,000 population) in 2018 (EFSA and ECDC, 2019). Nevertheless, the majority of human cases is thought to be sporadic, with a low fatality rate (0.03% in EU in 2018) (EFSA and ECDC, 2019). Indeed, within 220,682 confirmed human cases in 2019, only 18 outbreaks with strong-evidence were notified. Furthermore, based on the data reported separately to EFSA and ECDC, an estimation suggested that only 0.6% of human cases would be reported through food-borne outbreaks investigation (EFSA and ECDC, 2021a). Campylobacteriosis reporting is different according to surveillance systems for each country. The notification of the disease is mandatory for most European Union Member States (EU MS), including Luxembourg since 2020 (Gouvernement du Grand-Duché de Luxembourg, 2019). Consequently, interpretation of the data and comparison between countries should be carefully performed as the reported cases of *C. jejuni* and *C. coli* human infections are likely to represent only the tip of the iceberg owing to underestimation.

In Luxembourg, an integrated surveillance of *C. jejuni* genetic profiles has been implemented at the national level since 2005, by collecting isolates from diverse sources and by characterizing them by the MLST method (Ragimbeau et al., 2008). Then, an extended MLST method was developed by including two additional molecular markers, *gyr*A and *por*A loci (Ragimbeau et al., 2014). Through its application, the temporal distribution of the MLST+ profiles (ST-*gyr*A-*por*A) collected from human clinical isolates (N = 1,158 samples) and isolated between 2011 and 2012 was investigated. It has been revealed that 20 MLST+ profiles (3% in the whole collection of profiles) appeared regularly over years and almost 30% of the patients were infected by them. This phenomenon is intriguing as *C. jejuni* infections are mainly considered as sporadic, as previously mentioned. Indeed, if half of the human cases were neither sporadic nor epidemic, would they be part of diffuse outbreaks?

The first main part of this research work was to elucidate the genetic population structure of *C. jejuni* in Luxembourg. The strategy was to examine different genotypes, i.e., extended-MLST genetic profiles, at the core genome and whole genome levels by using comparative genomics methods. One of microbial population genetics aims is to describe and measure genetic diversity and its variability over time. It also determines the genetic distance between individuals and allows the analysis of how the diversity has arisen through the evolution of the genome composition (e.g., mutations, gene loss or acquisition, and homologous recombination). Indeed, all these genetic components and their dynamics shape the structure of the bacterial population. This particular type of study on evolutionary process has been carried out for about thirty years. Maynard Smith *et al.* (1993) described different types of bacterial population organization based on MLEE results. They demonstrated that the structure could range from clonal, with no recombination either between isolates in the same or different branches of the trees, to panmictic structure, with frequent and random recombination between isolates. An intermediate structure was described, in which recombination do not occur between isolates from two different major branches but are frequent between isolates within a major branch (Maynard Smith et al., 1993). Based on this concept, studies performed deep sequencing analyses to determine population structure of diverse bacterial pathogens, such as *Cyanobacteria* and *Streptomyces flavogriseus*. Both species population structure were found to approach panmixia (Doroghazi and Buckley, 2010; Rosen et al., 2015). In contrast, *Pseudomonas aeruginosa* was frequently considered as recombining, with a structure near to the panmictic one, in the past. However, a recent study highlighted that the majority of isolates are segregated into two distinct groups (Groups A and B) (Ozer et al., 2019), with frequent intragroup but limited intergroup recombination, corresponding to the intermediate structure. In the same way, the population structure of *Escherichia coli* was well studied over the past decades, and a high level of recombination was frequently highlighted. However, it did not destroy *E. coli* clonal population structure (Bobay et al., 2015).

A mutation rate was recently estimated for *C. jejuni* at 3.4×10^{-6} substitutions per site per year (s/s/y) (Calland et al., 2021). It is higher than the substitution rates of many other pathogens such as *Salmonella* Paratyphi A (clades 1 and 2; 1.2×10^{-7} s/s/y), *Mycobacterium leprae* (8×10^{-7} s/s/y), or *Yersinia pestis* (between 8×10^{-7} and 2.3×10^{-8} s/s/y) (Morelli et al., 2010; Duchêne et al., 2016; Calland et al., 2021). Despite this high level of mutations and homologous recombination acquired by HGT, Sheppard and colleagues have demonstrated that *C. jejuni* populations are highly structured into clusters of related isolates: the STs and CCs both derived from MLST data (Sheppard et al., 2012). The classification relies on grouping in the same genetic lineage, isolates that share identical alleles at four or more MLST loci with a

definable “central genotype” (Dingle et al., 2008; Colles and Maiden, 2012). Although *C. jejuni* and *C. coli* belong to the same genus, the *C. coli* species has a distinct population structure, including three different clades (clades 1 to 3). While clades 2 and 3 are more abundant in wildlife, waterfowl and the riparian environment, clade 1 share the same ecological niche than *C. jejuni*, mainly generated by intensive agriculture and animal production (Sheppard et al., 2011b). Strains classified within these clades are genetically more distant than strains classified within the CCs. However, the persistence of this 3-clade structure suggests that HGTs occur among members of the same clade and much more rarely between members of the different ones (Sheppard and Maiden, 2015). Indeed, the probability of genetic exchange decreases exponentially with the genetic distance between two strains (e.g., recombination within clades are more frequent than between clades for *S. enterica*) (Didelot et al., 2011). The level of HGTs is also influenced by many others factors such as ecological barriers which, as a result, keep the bacteria in different ecological niches apart (Calland et al., 2021). Asymmetric gene flow was identified among *C. jejuni* and *C. coli* clades 1, 2, and 3; clade 1 having the highest frequency of alleles originated in *C. jejuni* as they are facilitated by the share of the same ecological niche (Sheppard et al., 2008). Furthermore, genetic changes leading to the convergence of *C. coli* clade 1 with *C. jejuni* appeared to occur more frequently than those leading to their divergence did. Combined with further observations, these results led to the hypothesis that the two species are merging or “despeciating” (Sheppard et al., 2008). Recent identifications of extensive interspecies gene transfer from *C. jejuni* to *C. coli*, impairing differentiation and MLST analysis, support this hypothesis (Golz et al., 2020; Dieckmann et al.). In this context, the classification of *Campylobacter* strains, and consequently their traceability, is complex.

Therefore, defining the genetic relationship between *C. jejuni* isolates is quite challenging. Different strategies can be carried out to compare genomes, based either on SNP analyses or on gene-by-gene approaches. This latest method, commonly called cgMLST, appears to be more suitable to identify lineages with high recombining specie, such as *C. jejuni* (Sheppard et al., 2012; Dangel et al., 2019; Jajou et al., 2019). As presented in chapter II, several typing schemes based on the core genome or whole genome have been developed. According to the definition of the core genome applied and the panel of reference genome selected, the typing schemes include a different number of loci. Furthermore, the locus definition is also different within the available typing schemes. These variations potentially lead to different clustering. The classification of the strains could also vary within the typing scheme, as some have no defined cluster alert (e.g., the cgMLST Oxford and the wgMLST INNUENDO). The multiplication of possibilities to interpret the genomic data leads to the absence of a validated international nomenclature. It greatly complicates inter-laboratories communication in case of an outbreak, for instance. The

development and the harmonization of bioinformatics tools to perform these analyses are still under development. Indeed, in 2018, the European Commission requested an evaluation to set up a joint ECDC-EFSA database for the collection and analysis of WGS data, initially for *Listeria monocytogenes*, *Salmonella*, and *E. coli*. This should be extended to include other foodborne pathogens such as *Campylobacter* (EFSA and ECDC et al., 2019). Furthermore, PulseNet International also published its vision for the implementation of WGS for global food-borne disease surveillance. They critically reviewed technical and practical aspects of WGS for its use in public health laboratories to identify and to subtype food-borne pathogens (Nadon et al., 2017). However, there is still a need for a gold standard in the framework of epidemiological surveillance.

The recurrence in the *C. jejuni* genetic profiles in their temporal distribution could reflect the existence of diffuse outbreaks, which presumes that certain genomic lineages persist over space and time. In this situation, one might ask why this long-term survival exists. What could explain it? Would these clones have different adaptive abilities? The second part of this research study investigated whether particular phenotypical traits could be linked to the emergence and persistence of specific genotypes. The methodologies adopted consisted of phenotypical assays in controlled conditions and functional genomics analyses from wgMLST data. The objectives were to explore phenotypical features potentially linked to extracellular transmission, survival, and adaptation in the environment. Bacterial persistence is "the ability of a subset of the population to survive exposure to lethal stresses such as antibiotics" (Vogwill et al., 2016; Balaban et al., 2019). Wu *et al.* (2012) also suggested that these bacterial persisters' formation could be promoted by other stress responses than antibiotics, such as the oxidative stress induced by paraquat in *E. coli*, for instance (Wu et al., 2012). As exposed in chapter I, diverse phenotypical traits such as the survival to oxidative stresses, the acclimation to aerobic conditions, the adhesion to inert surfaces, and the biofilm formation capacities could contribute to environmental persistence. Typically, when these assays were performed, a phenotype profile characterizes each strain or lineage. Indeed, establishing the genetic basis of phenotype variation is among the most common objectives in microbiology. Thus, the second objective of this research work aimed to seek for a potential link between the recurrence of genomic lineages selected and their phenotypical traits. Understanding how genes content or changes in nucleotide sequences alter gene function and consequently affect phenotypical behavior is a major challenge. Then, in the era of functional genomics, the studies focus on identifying genes and on understanding their function and interactions to establish a relationship between the genotype and the organism phenotype (Kaushik et al., 2019).

2. Discussion of the results

In this second section, results obtained in chapters II, III, and IV are discussed regarding the technical and scientific questions.

2.1. Clonal expansion of stable genomes: *C. jejuni*, not only sporadic!

Population structure analysis is a major area of interest within many fields, such as genetics, epidemiology, and bioinformatics. It corresponds to the identification of shared genetic variations among strains and, accordingly, the classification of similar strains into clusters. Population structure analysis can be performed through several methods, categorized in two different approaches: parametric and non-parametric. Within the parametric ones, there is the Bayesian analysis of genetic population structure (BAPS), for instance. This approach identifies populations thanks to the genotype data based on the estimated allele or nucleotide frequencies for each population. It can assign strains to populations (Alhusain and Hafez, 2018). This method is based on a parametric approach, i.e., there is an important setting phase of the diverse parameters (e.g., a range of the number of populations *K*), and it requires intensive computational (Alhusain and Hafez, 2018). These two points have hindered the use of the BAPS method in this study, although it would have been of considerable interest to use it. However, non-parametric approaches have been designed to overcome the disadvantages of parametric approaches (Alhusain and Hafez, 2018). Therefore, a distance-based method was applied on our data, using pairwise similarities calculation to define genetic distances between isolates. The results are displayed through an allele difference (AD), used to reflect the genetic relationships between isolates. These values are then concatenated in a distance matrix, allowing the inference of the population structure according to the clustering approach chosen (e.g., Neighbor-joining tree-based clustering) (Limpiti et al., 2014). The phylogenetic approaches, based on WGS data, are known as core or whole genome MLST. Three cgMLST and one wgMLST typing schemes were selected for this study and their concordance was assessed. Overall, a high concordance in clustering strains was observed between the three cgMLST typing schemes (cgMLSTs Oxford, INNUENDO, and SeqSphere+) (chapter II). These results reinforced the decisions taken by the European Union and PulseNet to use a gene-by-gene approach and the cg/wgMLST typing scheme analysis (Nadon et al., 2017; EFSA and ECDC et al., 2019).

Moreover, the clonality signal appearing through the concordance of the different typing schemes in classifying strains supports the idea of stability of these clones over time and sources (chapter II). The definition of clonality is sometimes a matter of controversy, with scientists having different views on its stringency. Indeed, some authors distinguish “strict clonality” (mitotic propagation) and situations where recombination are restricted, and other authors define clonality by restrained recombination only. Tibayrenc and Ayala (2017) uphold the latter definition in their concept of predominant clonal evolution (PCE) (Tibayrenc and Ayala, 2017). One of the main features of their model is that recombination is not frequent enough (i.e., restrained on the evolutionary scale) to break up the prevalent pattern of the clonal population. Although the rate of nucleotide change (point mutation) is substantial in *C. jejuni*, the recombination is the major source of sequence variation and it drives the genetic evolution of *C. jejuni* (Calland et al., 2021). Despite this source of variation, and according to the work of Calland and colleagues on the *Campylobacter* molecular clock (2021), a significant proportion of *C. jejuni* lineages (22%) have been maintained for over four decades. In contrast, only 6% of the lineages were present in the *C. coli* population 40 years ago.

The persistence in time of some lineages could be explained by the fact that (i) the bacteria occupy specific ecological niches and are therefore not in direct competition; and (ii) the differences in fitness between the strains are not sufficient for one lineage to out-compete another (Calland et al., 2021). Therefore, it is possible that a limited number of recombination have allowed the persistence of specific lineages such as the four identified in this research. Indeed, for instance, lineage A (CC ST-21, ST-19) is endemic and has appeared regularly over more than a decade (chapter II). Furthermore, as described by Shapiro *et al.* (2016), natural pressure of selection has a significant influence on the bacterial population structure as it is considered as a force that promotes clonal expansions of adapted variants within an ecological niche (Shapiro, 2016). This fits with one of the plausible explanations for the lineage persistence from Calland *et al.* (2021) (Calland et al., 2021). Interestingly, one of the studied lineage, lineage B, belongs to the CC ST-257 and is thus considered as a chicken specialist. The ecological selection has certainly promoted the prevalence of this lineage in the poultry reservoir up to its predominance as an evolution process. However, the presence of persistent lineages, and in the context of our study the identification of four of them, leads to many questions. Are we moving towards the emergence of *C. jejuni* clones adapted to our industrial animal production? This phenomenon has previously been observed with others pathogens, such as *Salmonella*. In recent years, a shift in their serotypes related to poultry farming has been reported in diverse geographical regions, being particularly associated with the spread of certain well-adapted clones (e.g., *S.* Infantis and *S.* Stanley, for instance) (Antunes et al., 2016). Is this intensive

animal production an “incubator” for these clones? Mourkas *et al.* (2020) demonstrated the process by which one *C. jejuni* lineage, the CC ST-61, has undergone host specialization and became a cattle-associated lineage through agricultural intensification and, more precisely, intensive livestock production (Mourkas et al., 2020). They suggested that a progressive step-wise differentiation occurred at the generalist CC ST-21 (e.g., some accessory gene gains are linked to cattle adaptation) and led to the emergence of new clones circulating globally within cattle (Mourkas et al., 2020).

2.2. Diversity of phenotypical responses within a clonal population and introduction of the metaphenotype approach

The phenotypic tests were performed on the whole collection, including the strains related to the four genomic lineages recurrently identified in human infection, as well as on an outgroup panel corresponding to sporadic strains. A high diversity in the phenotypical responses appeared within the different genomic lineages. Indeed, lineages A and B were significantly more resistant to paraquat stress than lineages C and D, for instance. Strains belonging to lineage B had a resistance to hyperoxide stress significantly higher than strains from lineages C and D. Furthermore, each lineage had an adhesion significantly different from the three others, although lineages C and D had an adhesion significantly higher than lineages A and B. Finally, lineage D displayed a biofilm-forming capacity significantly higher than lineages A, B, and C. Concerning the acclimation to aerobic conditions, no statistical correlation could have been performed; however, a trend for acclimation to aerobiosis was observed for lineages A and D. Surprisingly, except the intra-test correlations, no correlation was found between the phenotypical responses. It was previously demonstrated that aerotolerance contributes to the defence against oxidative stress, as exposure to oxygen results in ROS accumulation (Oh et al., 2015a). Although the acclimation to AC requires an adaptation capacity not needed in aerotolerance (Demmig-Adams et al., 2008), the hypothesis was that a strain able to cope with oxidative stresses would be able of acclimation to aerobic conditions, and vice versa. However, lineages A and B were resistant to the oxidative stresses, and only lineage A tends to acclimate to AC. Furthermore, strains from lineage D, displaying a tendency for this acclimation, did not survive after oxidative stresses exposure. Along the same line, an enhanced biofilm development was demonstrated under aerobiosis (Sulaeman et al., 2012; Turonova et al., 2015), leading to a second hypothesis: strains able to develop a biofilm would acclimate to AC, and vice versa. However, lineages A and D tend to be acclimated to AC, and only lineage D was classified as a high-biofilm former. Thus, the genomic lineages could not be segregated according to only one phenotype. However,

the study revealed that a genomic lineage could be characterized by a combination of independent phenotypes, called here metaphenotypes. Our study then demonstrated the presence of monomorphic lineages over time that were characterized by a metaphenotype.

Traditional phenotypical studies of single traits may be unable to detect a global phenotypical response. However, phenotypes are often related to global responses induced by the regulation and expression of multiple genes, being then translated into proteins forming themselves a functional unit. The metaphenotypes integrate more information regarding the underlying biological process. Thus, it is possible to take advantage of these metaphenotypes to identify genetic elements influencing them, and therefore influence the survival and persistence of the strains in the environment.

2.3. Surprising ability to acclimate to aerobic conditions

In our collection, lots of strains demonstrated a capability to acclimate to aerobic conditions, with some even being able to grow under fully aerobiosis atmosphere. This feature is not as rare as expected. As previously mentioned, some lineages could develop particular phenotypical adaptations. Although it remains to be confirmed, in this study, strains belonging to lineages A and D tend to be more acclimated to aerobic conditions than strains in lineages B and C. A part of the acclimated colonies was stored at -80°C in the standard conditions, and none of them was able to grow again when directly submitted to aerobiosis. It was highlighted that the acclimation for these strains could be recovered only by using the same acclimation protocol with a first passage in microaerobic conditions.

In chapter III, WGS data from 6 out of 10 strains before and after their acclimation to AC were analyzed with the cgMLST Oxford scheme (1,343 targets). According to the results, four strains were identical in alleles before and after acclimation to AC, whereas one difference in allelic profile was recorded for Camp022 and Camp098. This difference is due to an amino acid change in the Cj0276 locus, also named *mreB*. MreB is involved in the shape transition of *C. jejuni* from rod to coccoid shape (Chiu et al., 2008). Previous work shown also that the transcript level of *mreB* was lower in *C. jejuni* Bf acclimated to aerobiosis and harboring mainly coccoid shapes (Rodrigues et al., 2015). The amino acid transition observed from the point mutation of *mreB* after acclimation might not affect its property. As this allelic difference does not appear in the other acclimated strains tested, we assumed that it may not be necessary for acclimation to AC. A SNP comparison could have been done to determine the potential shifts in homopolymeric tracts, for instance. However, as an acclimation step is required for the growth and

multiplication in AC, it suggests that the acclimation mechanisms are not acquired at the genetic level but are certainly dependent on regulations either at the gene, transcript or protein levels. Correlatively to the presence of DNA methylases, acclimation could be a result of epigenetic regulation mechanism.

In our study, the correlation between phenotype and gene specifically present in specific lineages gives us prospective markers rather than a definite answer. The functional analysis revealed the presence of methyltransferases and the *hsdS* gene (i.e., type I restriction-modification system, subunit S) within the unique targets identified in lineage A. Is there a potential link between the presence of specific methyltransferases and the diversity of phenotypic responses to acclimation to AC? Epigenetics (i.e., the study of phenotype changes that do not result from modifications in the DNA sequence) most often involves changes that affect gene expression. DNA methylation is widely distributed in bacteria and is the only known mechanism by which prokaryotes could modify their gene expression. Furthermore, methylation studies have significantly advanced since the advent of NGS technologies, such as the single-molecule real-time sequencing, which enables the detection of the three major forms of bacterial DNA methylation (Beaulaurier et al., 2019). They are of great concern as the emergence of epigenetic lineages enables the adaptation of bacterial populations to harsh environments and moderates their behavior (Sánchez-Romero and Casadesús, 2020). For instance, a study found phenotypical evidence supporting the hypothesis that *C. jejuni* methyltransferases play a regulatory role in phenotypes, such as motility and adhesion (Kim et al., 2008). Mou *et al.* (2015) compared three *C. jejuni* methylome profiles: NCTC 11168, 81-176, and the SA clone (Mou et al., 2015). They detected hypo and hyper-methylated regions in their genomes, and they demonstrated that restriction-modifications (RM) activities may play a role in the gene expression and might be correlated with the hypervirulence phenotype of the SA clone (Mou et al., 2015). RM systems are often considered as primitive bacterial immune systems; however, they are also involved in the DNA mismatch repair and the regulation of gene expression (Vasu et al., 2019). Indeed, studies revealed that they have a putative role in regulating several virulence genes (e.g., a flagella gene *flhB* and a RNA polymerase sigma factor *rpoN*) (Ghatak et al., 2020). Thus, the combined action of the methyltransferases and the RM system identified in genomic lineages could be an explanation to enhance the diversity of phenotypical responses, particularly to potential acclimation to AC in the lineage A strains, for instance.

Phenotypical responses could also be regulated at the transcriptomic or proteomic levels. As proteomics encompasses the global analysis of proteins expression at the organism level, this type of analysis was planned to understand how the bacteria respond to changes in the environment, or for

mapping subcellular locations of proteins, for instance. It was already demonstrated on *C. jejuni* Bf strain, that the transcript levels of KatA, TrxB, and SodB was higher after the acclimation to AC as compared to cultivation in aerobiosis without acclimation (Rodrigues et al., 2016), indicating that specific proteins might be specifically regulated for acclimation mechanism. Does the upregulation could be observed in other aerobiosis acclimated strains? Correlatively, a higher catalase-equivalent efficiency in the Bf strain than in the NCTC11168 strain in MAC, while no difference was observed between MAC and AC for the Bf strain. These results led to the hypothesis that the baseline efficiency of the enzyme might be higher in certain strains. Furthermore, a difference was observed between AC and AAC: the catalase-equivalent efficiency was slightly higher in AAC than in AC in the Bf strain (Rodrigues et al., 2016). Is this improved efficiency due to a higher pool of enzymes or to an enhanced efficiency of the enzyme? Altogether, these results indicate that acclimation-specific posttranscriptional and posttranslational mechanisms take place in acclimated strains: an atypical acclimation could result from multifactorial events. However, further results are needed to better understand this mechanism.

To do so, an exploratory study based on a holistic approach at the level of protein expression was initiated to identify cellular, molecular, and regulatory mechanisms underlying the acclimation to AC. A collaboration was contracted with the Luxembourg Institute of Health (LIH), which is developing a high-throughput proteomic sequencing on small protein samples to get a proof-of-concept in the microbiology domain. Indeed, this technique has never been used on prokaryotic organisms before. This proteomic analysis allowed the fast sequencing of all the peptides (e.g., 5 minutes per sample), leading to the protein sequence and the post-translational modifications identifications, to the proteome mapping, and to the relative (fold change) and absolute (concentration) quantifications. During my thesis, we designed a scientific workflow to represent the different conditions with technical and biological replicates of several strains with or without the ability to grow and acclimate to aerobic conditions. A total of 70 strains were cultured in up to three biological and technical replicates. According to the workflow, samples were collected in a specific cell-lytic buffer and sent to the collaborator for proteomic analysis. The analyses are still in progress at LIH, and the future results aim at determining the metabolic pathway involved in cell acclimation. Unfortunately, the delay due to the health crisis could not end up with results up to date.

2.4. Potential molecular markers revealed through functional genomics analysis

Through the wgMLST analyses and the identification of unique targets in the four lineages, research of possible links between the unique targets identified and the phenotypical responses was performed. As described by San *et al.* (2020), microbial genome-wide association studies (mGWAS) are a suitable technique to analyze how variations in microbial genomes affect pathogen phenotypes (San et al., 2020). For instance, GWAS identified vitamin B_5 biosynthesis as a host specificity factor for cattle gut colonization by *C. jejuni* (Sheppard et al., 2013). It also allowed the identification of 25 genes as putative diagnostic markers for human pathogenic strains (Buchanan et al., 2017). Furthermore, GWAS also provided evidence of functional traits linked with *C. jejuni* survival from farm to fork (Yahara et al., 2017). A genome-wide identification of host-segregating SNPs for source attribution was also performed on clinical *C. coli* isolates (Jehanne et al., 2020). However, several computational tools and methods have been developed, but no comprehensive, end-to-end, and user-friendly tool is currently available (San et al., 2020). Furthermore, the presence of multiple isolates of clonal background could reduce the power of this technique, according to Sheppard *et al.* (2013) (Sheppard et al., 2013). Added to the fact that a very high number of samples is required to reach sufficient power, this technique could have been used if the strain collection had been expanded. However, an alternative strategy was favored, which aimed to focus mainly on phenotypically characterized strains.

As discussed in chapter IV, very interesting targets were highlighted, such as 12 core components of the T6SS potentially linked to the metaphenotypes of lineages C and D. The 13th core component of the T6SS, the TssH protein, also named ClpV, was not found in any *C. jejuni* strain as previously described (Robinson et al., 2021). However, the RloA ATPase, a potential alternative to ClpV, was identified in all the strains of lineage D only. The combination of the 12 core components of the T6SS and the RloA might form a complete and functional T6SS contributing to explain the metaphenotype of lineage D. As this RloA protein and the 12 core components of the T6SS have not been identified together in any other strain in our collection, this unique combination could be specific of strains belonging to lineage D. It would be interesting to investigate a large public database such as PubMLST to screen available genomes toward the 12 core components of the T6SS and the RloA locus (Jolley et al., 2018). This analysis would make it possible to check if this unique combination of targets forms a specific molecular marker to distinguish the lineage D. Furthermore, if these 13 targets are identified in several strains, it could be of interest to perform the adhesion and biofilm formation tests on them. Indeed, it has been demonstrated that the

T6SS could enhance the biofilm formation in various species (e.g., in *Acinetobacter baumannii*) (Kim et al., 2017). The results of these tests may confirm the link between the presence of a potentially functional T6SS and the ability to form biofilms.

This potential molecular marker needs to be confirmed by further analyses. However, its presence within the specific lineage D, whose strains displayed particular adaptive phenotypical responses, reinforce the assumption that phenotypical abilities contribute to the persistence and the transmission in the environment over years.

3. Challenges and outlooks

Next-generation sequencing (NGS) provides rapid, economical, and high-throughput access to bacterial whole genomes sequences. This state-of-the-art technology offers significant advantages, such as the possibility to use only one method per pathogen to obtain in one run a large amount of genomic data and a wide range of subsequent analyses. Different bioinformatics pipelines are now available for typing, for the virulome or for determining antimicrobial resistance. Concerning the cost, it is less expensive and less labor intensive than MLST Sanger-based method for *Campylobacter*, for instance (ECDC, 2015; Alleweldt et al., 2021). Furthermore, the discriminatory power of nucleic acid sequencing methods is higher than the one of other molecular typing methods (e.g., MLST), which leads to the opportunity to explore more accurately the phylogenetic relationship of bacterial isolates and allows more robust case identifications in outbreak investigations (Koutsoumanis et al., 2019). Concerning the timeliness, the total time required for this technique seems to be comparable to other typing methods. However, the time remains a challenge in the clinical field: the week it can take for WGS data generation and analysis has to be reduced to mere days, requesting a continuous improvement of this technique. In this sense, rapid advances at the NGS platforms level (e.g., automation of the WGS library preparation) have made the application of WGS in the context of outbreak events possible, such as for the outbreak detection of verocytotoxin-producing *Escherichia coli* (VTEC) (Joensen et al., 2014; Besser et al., 2019). Along the same lines, in 2019, ECDC implemented a strategic framework for integrating molecular and genomic typing into European surveillance and multi-country outbreak investigations (ECDC, 2019b). ECDC proposed to prioritize the implementation of WGS, depending on the disease and public health application. The WGS is used for continuous surveillance for influenza virus, *Listeria monocytogenes*, multidrug-resistant *Mycobacterium tuberculosis*, *Neisseria meningitides*, *Salmonella enterica*, and Shiga-

toxin producing *E. coli*. While it is only used in an outbreak investigation objective for *Campylobacter* sp., *Clostridium difficile*, *Listeria monocytogenes*, *Salmonella enterica*, and Shiga-toxin producing *E. coli*, for instance (ECDC, 2019b).

However, without continuous surveillance, detecting diffuse outbreaks of *C. jejuni* (i.e., clusters of cases with a suspected common source, which are not necessarily clustered geographically nor through an epidemic curve) is particularly challenging, as these cases are concealed by the multiple sporadic cases (Llarena et al., 2017). Diffuse outbreaks of campylobacteriosis were investigated in earlier studies, and their higher recurrence was demonstrated (i.e., several times more common than point-source outbreaks) (Jonsson et al., 2010; Strachan and Forbes, 2016). Through this work, waves of certain lineages that emerged and persist over time were revealed, which is very different from the other foodborne pathogens where clear epidemic peaks are usuallpiloy observable, such as *Listeria monocytogenes* (Thomas et al., 2020). Therefore, it would be worth implementing continuous surveillance for *C. jejuni*, the diffuse outbreak detection requiring long-term data (e.g., 13 years in our study). A first step in this implementation could be a one year-pilot study at the European level. Indeed, both epidemic signals and diffuse outbreaks could be detected more effectively by large-scale surveillance than in a small area like Luxembourg.

In the latest EFSA/ECDC reports, the number of foodborne illnesses seems to be slightly decreasing which might suggest the irrelevance of the implementation of a continuous surveillance (EFSA and ECDC, 2021a). However, one should not be misled by the decrease of these data. Indeed, it could be linked to the COVID-19 pandemic as Mughini-Gras *et al*. (2021) recently exposed. In their study, the impact of the global pandemic on the human salmonellosis incidence in the Netherlands resulted in a decrease by over 50% compared to the 2016-2019 period (Mughini-Gras et al., 2021). Furthermore, according to the New Zealand Food Safety report, reporting rates for most foodborne diseases were lower (e.g., for *Campylobacter* and *Salmonella*) due to the global sanitary situation (Ministry for Primary Industries, 2021). Indeed, the repeated lockdowns imposed in many countries have led to the closing of restaurants and the restriction of travel, thereby reducing the risk of exposure. In addition, at the EU level, one member state notified EFSA and ECDC that the reported number of campylobacteriosis cases was lower than expected, as data were not received from all regions due to the COVID-19 pandemic in 2020 (EFSA and ECDC, 2021a).

In a survey in 2020, almost half of the member states have reported the capacity to perform WGS on *Campylobacter* isolates (EFSA and ECDC, 2021a). Furthermore, WGS was implemented in a huge

number of laboratories for the SARS-CoV-2 sequencing. Indeed, its sequencing is strongly promoted by ECDC and WHO as it supports the monitoring of the disease's spread and evolution of the virus (WHO, 2021; WHO and ECDC, 2021). Therefore, the equipment is widely available now in laboratories. In addition, although it is amplicon sequencing in the framework of SARS-CoV-2 sequencing, the technicians have been trained in sequencing technology and will be able to adapt their knowledge by following protocols for bacterial sequencing; thus, it would be possible to easily implement continuous surveillance of *Campylobacter*.

The primary reservoir of *C. jejuni* is broiler related. However, Luxembourg has a negligible production and most of the chicken consumed is imported from neighboring countries. The recurring lineages identified in Luxembourg could thus be cross-borders and geographically spread out. Indeed, previous studies already highlighted the dispersion through travelers (CC ST-464 in the USA) or potentially through wild birds (Kwan et al., 2014; Cha et al., 2016; Kovanen et al., 2019; Shrestha et al., 2019). Furthermore, through a collaboration with another member state, strains with the same genotype as the strains belonging to lineage A (ST 19) and which caused an epidemic event in their country of origin, were studied. It appeared that they were very close: 1 to 18 SNPs between these European strains and the Luxembourgish strains (personal communications inter-laboratories). First assays also demonstrated that these strains could acclimate to aerobic conditions, as well as lineage A strains in our collection (data not shown). These first insights confirm the need to implement long-term continuous surveillance for *C. jejuni* to monitor these recurrent lineages, to detect possible new clonal expansions, and to investigate the potential spreading of cross-borders clones. It will help improving prevention and control strategies at the European and national levels. For example, since these clonal lineages cause approximately 50% of human cases in Luxembourg, it might be the same proportion in other countries; thus, it might be worth considering the development of a vaccine against them. In the event of a new clonal expansion, the vaccine would be re-adapted in the same way as the seasonal flu vaccine is adapted each year (Centers for Disease Control and Prevention, 2021).

Through the exploration of a large collection of data, the strong interest in monitoring genotypes causing gastroenteritis was demonstrated, in the sense that campylobacteriosis is not only of sporadic nature. Behind the apparent huge genetic diversity of clinical isolates, the presence of recurrent genotypes has highlighted the persistence of stable clones representing a risk factor. Thus, the results of this thesis project gave new insights for the surveillance of *Campylobacter jejuni* infections, advising the implementation on a continuous surveillance at the European level in a framework of prevention efforts

to reduce the burden of *C. jejuni* in foodborne diseases. Furthermore, this thesis scaled-up the knowledge of phenotypical traits at the strain collection level. The results brought new insights about the distribution of specific phenotypical features involved in the persistence and survival of *Campylobacter jejuni* in the environment. Indeed, the underlying molecular mechanisms for transmission, persistence, and survival were assumed to be connected. However, there was no inter-dependency between the phenotypical responses, and each recurrent genomic lineage was characterized by a specific metaphenotype. Furthermore, based on these metaphenotypes and through functional genomics analysis, a potential molecular marker, i.e., the combination of the 12 core components of the T6SS and the RloA, was revealed. It could distinguish strains belonging to the lineage D and able to develop a biofilm. In addition, the ability of *C. jejuni* strains to infect chickens is enhanced by the presence of the T6SS (Liaw et al., 2019). Veterinary authorities could therefore assess the prevalence of the T6SS within poultry isolates. Finally, outbreaks represent a significant risk to patient safety and their investigations are costly and time consuming. The implementation of targeted surveillance of specific genomic lineages could provide key information to develop additional prevention and control measures for *C. jejuni* infections, since half of human cases may be related to them.

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Appendices

Appendix A: Strain panel, source and date of isolation, lineage and phenotypical results.

Sample ID	Source	Year of isolation	Lineage	CC	PQ stress - survival rate (log_{10} ufc/mL)			H_2O_2 stress - survival rate (log_{10} ufc/mL)			Aerobic acclimation	Adhesion (average ΔBFI)	Biofilm (average ΔBFI)	Antimicrobial resistance
					0.12 mM	0.25 mM	0.50 mM	0.12 mM	0.25 mM	0.50 mM				Fluoroquinolones
Camp001	MA	2005	A	ST-21	0.78	0.67	0.32	0.58	0.39	0.00	NAAC	6.83	0.67	R
Camp005	H	2006	A	ST-21	0.88	0.68	0.49	0.58	0.56	0.37	NAAC	7.52	0.61	R
Camp003	H	2006	A	ST-21	0.59	0.38	0.00	0.59	0.41	0.00	SAAC	6.80	3.63	R
Camp004	MA	2006	A	ST-21	0.79	0.64	0.36	0.45	0.00	0.00	SAAC	7.00	1.12	R
Camp006	H	2007	A	ST-21	0.60	0.38	0.00	0.32	0.00	0.00	NAAC	2.35	1.55	R
Camp007	V	2011	A	ST-21	0.60	0.29	0.00	0.47	0.29	0.00	SAAC	7.29	4.71	S
Camp010	MA	2011	A	ST-21	0.65	0.42	0.00	0.46	0.34	0.00	SAAC	1.43	1.17	R
Camp008	SW	2011	A	ST-21	0.58	0.29	0.00	0.47	0.31	0.00	SAAC	6.21	2.67	R
Camp009	H	2011	A	ST-21	0.81	0.66	0.27	0.35	0.00	0.00	NAAC	11.00	1.17	R
Camp011	SW	2012	A	ST-21	0.73	0.35	0.00	0.33	0.00	0.00	SAAC	0.89	1.96	R
Camp014	H	2012	A	ST-21	0.67	0.35	0.00	0.55	0.36	0.00	SAAC	1.63	1.64	R
Camp015	MA	2012	A	ST-21	0.41	0.00	0.00	0.48	0.36	0.00	NAAC	2.72	0.80	R
Camp016	V	2013	A	ST-21	0.54	0.30	0.00	0.47	0.25	0.00	NAAC	3.81	2.74	R
Camp018	H	2013	A	ST-21	0.56	0.30	0.00	0.47	0.00	0.00	AAC	7.04	1.39	R
Camp017	H	2013	A	ST-21	0.58	0.23	0.00	0.59	0.37	0.00	AAC	6.53	3.45	R
Camp021	MA	2014	A	ST-21	0.62	0.44	0.00	0.00	0.00	0.00	NAAC	2.07	1.72	R
Camp019	H	2014	A	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	2.69	1.16	R
Camp023	MA	2015	A	ST-21	0.65	0.50	0.00	0.46	0.00	0.00	AAC	5.29	1.30	R
Camp028	H	2016	A	ST-21	0.51	0.00	0.00	0.33	0.00	0.00	SAAC	1.16	2.29	R
Camp035	H	2017	A	ST-21	0.78	0.49	0.24	0.58	0.46	0.00	SAAC	1.62	0.75	R
Camp034	H	2017	A	ST-21	0.56	0.39	0.00	0.33	0.00	0.00	NAAC	1.38	0.00	S
Camp036	MA	2018	A	ST-21	0.55	0.00	0.00	0.00	0.00	0.00	AAC	11.44	2.04	R
Camp039	H	2014	B	ST-257	0.32	0.00	0.00	0.32	0.00	0.00	NAAC	3.44	0.80	R
Camp041	V	2014	B	ST-257	0.45	0.29	0.00	0.63	0.39	0.00	NAAC	1.96	0.56	R
Camp040	H	2014	B	ST-257	0.65	0.35	0.00	0.76	0.58	0.00	SAAC	0.47	1.38	R
Camp045	V	2015	B	ST-257	0.51	0.32	0.00	0.67	0.51	0.00	NAAC	0.65	0.24	R
Camp043	H	2015	B	ST-257	0.83	0.64	0.42	0.54	0.24	0.00	NAAC	1.48	0.87	R
Camp047	H	2015	B	ST-257	0.62	0.41	0.27	0.39	0.00	0.00	NAAC	0.55	0.63	R
Camp049	MA	2016	B	ST-257	0.65	0.29	0.00	0.59	0.36	0.00	NAAC	3.04	0.35	R
Camp050	H	2017	B	ST-257	0.77	0.60	0.51	0.42	0.00	0.00	NAAC	1.50	1.21	R
Camp053	H	2018	B	ST-257	0.42	0.00	0.00	0.48	0.36	0.00	NAAC	1.35	0.61	R
Camp052	H	2018	B	ST-257	0.60	0.33	0.00	0.56	0.35	0.00	SAAC	1.57	1.05	R
Camp054	H	2012	C	ST-464	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	17.91	5.56	R
Camp055	V	2014	C	ST-464	0.00	0.00	0.00	0.34	0.00	0.00	NAAC	17.80	2.86	R
Camp059	V	2015	C	ST-464	0.77	0.56	0.00	0.36	0.29	0.00	NAAC	17.88	2.27	R
Camp057	V	2015	C	ST-464	0.35	0.00	0.00	0.41	0.00	0.00	NAAC	17.98	2.57	R
Camp063	V	2016	C	ST-464	0.31	0.00	0.00	0.31	0.00	0.00	NAAC	17.79	8.87	R
Camp064	V	2016	C	ST-464	0.22	0.00	0.00	0.36	0.00	0.00	NAAC	17.28	5.29	R

Sample ID	Source	Year of isolation	Lineage	CC	PQ stress - survival rate (log_{10} ufc/mL)			H_2O_2 stress - survival rate (log_{10} ufc/mL)			Aerobic acclimation	Adhesion (average ΔBFI)	Biofilm (average ΔBFI)	Antimicrobial resistance
					0.12 mM	0.25 mM	0.50 mM	0.12 mM	0.25 mM	0.50 mM				Fluoroquinolones
Camp069	MA	2017	C	ST-464	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	17.51	0.88	R
Camp071	H	2018	C	ST-464	0.47	0.29	0.00	0.46	0.00	0.00	NAAC	7.73	0.75	R
Camp075	V	2016	D	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	11.42	9.12	R
Camp079	H	2017	D	ST-21	0.34	0.00	0.00	0.35	0.00	0.00	NAAC	14.21	10.83	R
Camp082	V	2017	D	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	7.30	8.02	R
Camp078	H	2017	D	ST-21	0.00	0.00	0.00	0.46	0.00	0.00	SAAC	13.88	14.48	R
Camp077	H	2017	D	ST-21	0.42	0.00	0.00	0.00	0.00	0.00	AAC	8.78	9.13	R
Camp081	H	2017	D	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	14.45	9.89	R
Camp080	H	2017	D	ST-21	0.47	0.00	0.00	0.00	0.00	0.00	SAAC	15.79	15.17	R
Camp085	H	2018	D	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	17.34	13.37	R
Camp086	V	2018	D	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	16.05	14.25	R
Camp087	H	2018	D	ST-21	0.80	0.63	0.00	0.35	0.00	0.00	SAAC	12.87	12.71	R
Camp088	H	2018	D	ST-21	0.67	0.38	0.00	0.32	0.00	0.00	AAC	10.52	9.09	R
Camp089	H	2018	D	ST-21	0.69	0.46	0.00	0.00	0.00	0.00	AAC	7.58	10.43	R
Camp094	H	2010	UC	ST-48	0.60	0.42	0.31	0.40	0.00	0.00	NAAC	8.67	6.99	S
Camp105	H	2011	UC	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	8.20	6.21	R
Camp098	H	2011	UC	ST-206	0.78	0.66	0.27	0.30	0.00	0.00	AAC	18.16	13.90	S
Camp104	V	2011	UC	ST-353	0.45	0.00	0.00	0.32	0.00	0.00	SAAC	17.44	15.96	S
Camp107	H	2011	UC	ST-48	0.42	0.30	0.00	0.61	0.00	0.00	NAAC	16.67	14.73	S
Camp091	H	2011	UC	ST-21	0.29	0.00	0.00	0.43	0.00	0.00	NAAC	13.19	1.53	R
Camp101	H	2011	UC	ST-354	0.40	0.00	0.00	0.54	0.44	0.00	NAAC	16.05	14.61	R
Camp097	H	2011	UC	ST-21	0.00	0.00	0.00	0.52	0.25	0.00	NAAC	8.05	4.68	S
Camp092	H	2011	UC	ST-42	0.00	0.00	0.00	0.00	0.00	0.00	AAC	7.80	7.72	S
Camp102	H	2011	UC	ST-607	0.35	0.00	0.00	0.00	0.00	0.00	SAAC	14.31	11.33	R
Camp106	H	2011	UC	ST-21	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	5.59	6.64	R
Camp095	H	2011	UC	ST-48	0.76	0.64	0.43	0.85	0.79	0.55	SAAC	17.53	15.36	R
Camp090	H	2011	UC	ST-21	0.75	0.66	0.50	0.88	0.79	0.66	SAAC	18.14	13.56	S
Camp108	H	2011	UC	ST-45	0.42	0.00	0.00	0.27	0.00	0.00	SAAC	7.62	5.69	R
Camp100	H	2011	UC	ST-21	0.44	0.26	0.00	0.57	0.43	0.26	SAAC	17.25	8.46	S
Camp093	H	2011	UC	ST-21	0.70	0.37	0.00	0.46	0.00	0.00	SAAC	1.60	0.82	R
Camp103	H	2011	UC	ST-21	0.76	0.48	0.33	0.70	0.55	0.32	SAAC	11.33	4.26	S
Camp096	H	2011	UC	ST-48	0.80	0.66	0.57	0.86	0.69	0.44	NAAC	17.70	14.03	S
Camp099	H	2011	UC	ST-257	0.60	0.40	0.35	0.92	0.65	0.23	SAAC	13.41	3.62	S
Camp112	SW	2011	UC	ND	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	17.41	10.61	S
Camp113	SW	2011	UC	ST-952	0.36	0.00	0.00	0.00	0.00	0.00	NAAC	2.63	1.30	S
Camp114	SW	2011	UC	ND	0.66	0.48	0.38	0.52	0.28	0.00	SAAC	12.49	11.57	S
Camp115	SW	2012	UC	ND	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	11.04	9.01	R
Camp116	SW	2012	UC	ND	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	3.81	1.27	S

Sample ID	Source	Year of isolation	Lineage	CC	PQ stress - survival rate (log_{10} ufc/mL)			H_2O_2 stress - survival rate (log_{10} ufc/mL)			Aerobic acclimation	Adhesion (average ΔBFI)	Biofilm (average ΔBFI)	Antimicrobial resistance
					0.12 mM	0.25 mM	0.50 mM	0.12 mM	0.25 mM	0.50 mM				Fluoroquinolones
Camp111	SW	2012	UC	ND	0.63	0.44	0.00	0.48	0.35	0.00	SAAC	3.49	0.10	S
Camp117	SW	2012	UC	ND	0.00	0.00	0.00	0.00	0.00	0.00	SAAC	6.57	1.16	S
Camp109	SW	2012	UC	ND	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	4.59	0.74	S
Camp118	SW	2012	UC	ST-179	0.00	0.00	0.00	0.00	0.00	0.00	NAAC	3.91	1.12	S
Camp110	SW	2012	UC	ND	0.44	0.00	0.00	0.00	0.00	0.00	SAAC	5.75	0.33	S
Bf	H	1994	REF	ST-403	0.77	0.71	0.63	0.66	0.57	0.33	AAC	17.47	13.42	S
NCTC 11168 Lux	H	1977	REF	ST-21	0.75	0.46	0.00	0.68	0.45	0.00	SAAC	17.84	13.15	S

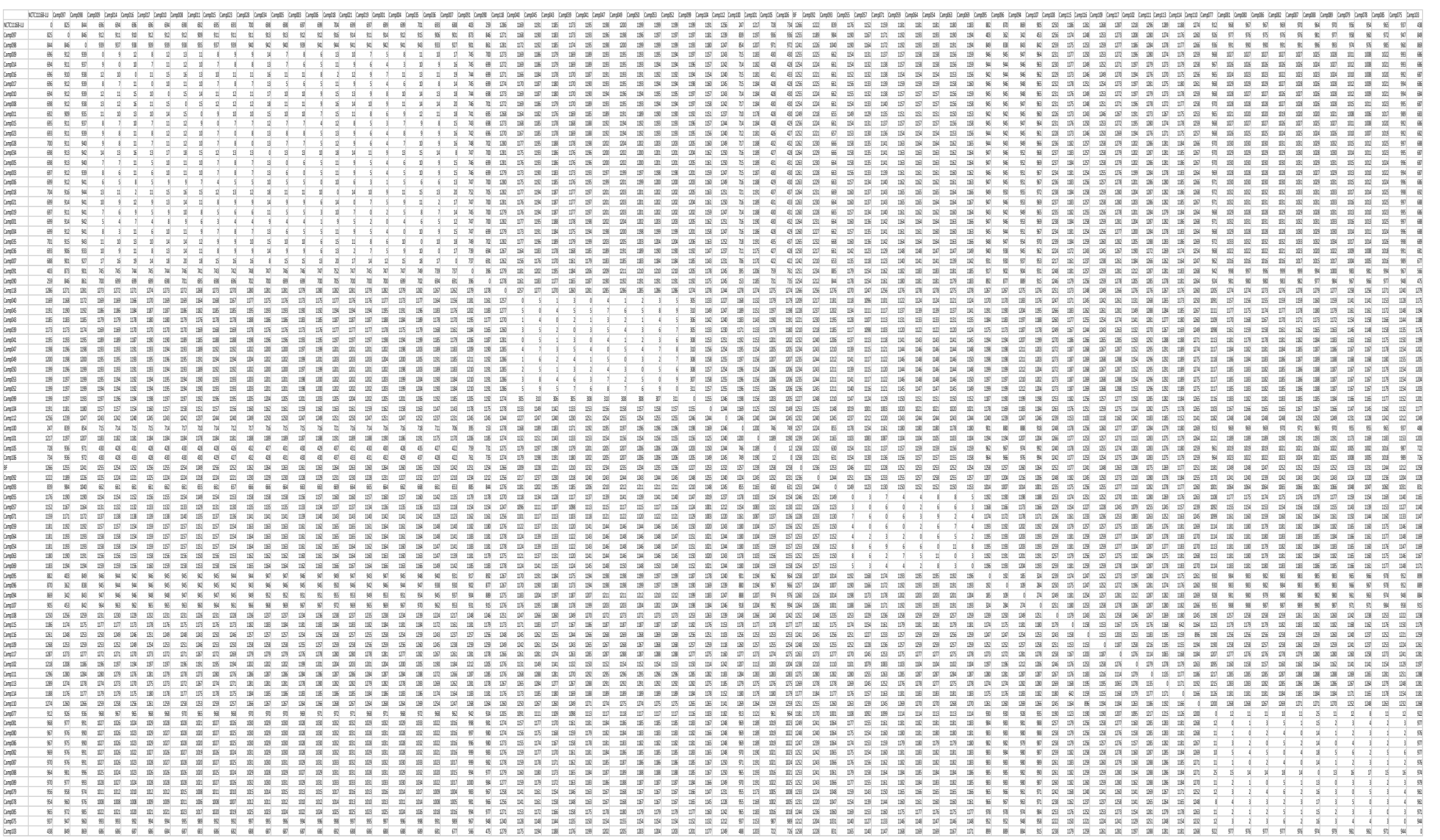
Source: H: human; MA: mammals; V: poultry; SW: surface water

UC: unique combination

Aerobic acclimation: NAAC: non-acclimated to aerobic conditions; SAAC: semi-acclimated to aerobic conditions; AAC: acclimated to aerobic conditions

Antimicrobial resistance: R: resistant to the antibiotic molecules; S: susceptible to the antibiotic molecules

Appendix B: Distance matrix analysis on the cgMLST Oxford analysis, on 83 *C. jejuni* strains.



DOCTORAT ECOLOGIE BRETAGNE GEOSCIENCES LOIRE AGRONOMIE ALIMENTATION

Oniris École Nationale Vétérinaire, Agroalimentaire et de l'Alimentation, Nantes Atlantique
Santé et alimentation au cœur de la vie

UNIVERSITÉ DU LUXEMBOURG

Titre : Structure de la population et traits phénotypiques de *Campylobacter jejuni* circulant au Luxembourg

Mots clés : *C. jejuni*, structure de la population, expansion clonale, persistance, métaphénotype

Résumé :
Campylobacter est la principale cause de gastro-entérite bactérienne mondiale. L'espèce la plus répandue, *C. jejuni*, est un pathogène microaérobie, capnophile et thermotolérant. De par ses exigences croissance, la capacité de *C. jejuni* à persister au sein des chaines de production alimentaire a longtemps intrigué les scientifiques.
Cette étude visait à comparer différents profils génétiques de *C. jejuni* isolés au Luxembourg, à partir du core genome (cg) et du génome entier (wg) afin de clarifier la structure génétique de sa population. En utilisant des tests phénotypiques en conditions contrôlées et des analyses de génomique fonctionnelle à partir de données wgMLST, le lien entre les traits phénotypiques et la persistance des génotypes a été exploré.
Le regroupement des souches en lignées génomiques est concordant avec des profils de type épidémique et endémique, indépendamment des schémas de typage cgMLST utilisés. La grande stabilité du génome au sein de ces lignées soutient l'hypothèse d'une expansion clonale de type monomorphique au fil du temps et de différentes sources. Les phénotypes sont corrélés aux complexes clonaux généralistes ou spécifiques à l'hôte pour les réponses au stress oxydatif, à l'adhésion aux surfaces abiotiques, à la formation de biofilms et à l'acclimatation aux conditions aérobies. Ces données ont permis d'établir des métaphénotypes spécifiques de ces lignées génomiques. L'analyse de génomique fonctionnelle a révélé des facteurs pouvant contribuer à la survie spatiotemporelle des souches récurrentes. Ces résultats suggèrent également une sélection de souches de *C. jejuni* mieux adaptées aux stress environnementaux le long des voies de transmission à l'Homme.

Title: Population structure and phenotypical traits of *Campylobacter jejuni* circulating in Luxembourg

Keywords : *C. jejuni*, population structure, clonal expansion, persistence, metaphenotype

Abstract :
Campylobacter is the leading cause of bacterial gastroenteritis worldwide. The most prevalent species, *C. jejuni*, is a strict microaerobic, capnophilic, and thermotolerant pathogen. Given its growth requirements, the ability of *C. jejuni* to persist in food environments and be transmitted throughout food processing has long puzzled scientists.
This study aimed to compare the different genetic profiles of *C. jejuni* strains, isolated in Luxembourg, at the core genome (cg) and whole genome (wg) levels to elucidate its genetic population structure. Using phenotypical assays in controlled conditions and functional genomics analyses from wgMLST data, the study also investigated the possible link between phenotypic traits and emergence or persistence of genotypes. A high concordance in strain clustering was observed between genomic lineage classifications and the epidemic and endemic signals, regardless the three cgMLST typing schemes used. The higher genome stability within genomic lineages supports the hypothesis of a clonal expansion with monomorphic patterns over time and sources. A high correlation was observed between phenotypes and host-specific or generalist clonal complexes for oxidative stress, adhesion to inert surfaces, biofilm formation, and acclimation to aerobic conditions responses. These data allowed the establishment of metaphenotypes specific to the genomic lineages. Functional genomic analysis revealed factors that may contribute to the spatiotemporal survival of recurrent strains. These results also suggest the selection of better-adapted and persistent *C. jejuni* strains to environmental stresses throughout the transmission route to human.